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A DISSERTATION
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IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY

BY
KARL KONRAD KOESSLER

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STUDIES ON PROTEINOGENOUS AMINES.

II. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING IMIDAZOLE DERIVATIVES.*

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(Received for publication, July 14, 1919.)

INTRODUCTION.

The method described below is based upon the interaction between the imidazole ring and *p*-phenyldiazonium sulfonate (*p*-diazobenzene sulfonate).[1,2] Although this reaction has been used for a long time as a qualitative test for imidazoles there has been, as far as known to the authors, but one attempt made to apply it quantitatively. Weisz and Ssobolew[3] claim that a maximum color is obtained with 1 mg. of histidine dichloride under the conditions specified by them. Quantities of histidine either larger or smaller than 1 mg. give less intense colors even when the amount of *p*-phenyldiazonium sulfonate is increased. No attempt was made by the above authors to apply their method to other imidazoles. The method which we shall describe has, we believe, the following advantages over that of the above authors.

(*a*) It can be applied directly to practically any imidazole derivative.

(*b*) It gives equally good results on mixtures or pure solutions of imidazoles.

(*c*) As little as 0.00001 gm. of any of the imidazoles can be estimated with a fair degree of accuracy.

* The first article of this series was published in *J. Am. Chem. Soc.*, 1918, xl, 1716.

[1] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[2] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[3] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1914, lviii, 119.

(*d*) A laborious series of dilutions is unnecessary. The first determination is usually a reliable index of the quantity of imidazole present.

In the ordinary qualitative procedure suggested by Ehrlich[1] and by Pauly[2] the imidazole solution is mixed first with the acid *p*-phenyldiazonium sulfonate reagent and this solution is then treated with an excess of a base, either sodium carbonate or ammonium hydroxide. We have found it necessary to mix the *p*-phenyldiazonium sulfonate solution first with the alkali sodium carbonate and then to add the imidazole-containing solution to this alkaline liquid to obtain strictly quantitative color productions. The necessity for such a change in procedure is readily seen when the chemical reactions involved are considered.

It is generally conceded that the first reaction proceeds according to the following equation.

$$(1)\quad HO-SO_2-C_6H_4-\underset{\displaystyle Cl}{\underset{|}{NH_3}}+HNO_2\rightarrow HO-SO_2-C_6H_4-\underset{\displaystyle Cl}{\underset{|}{N}}\equiv N+2H_2O$$

By allowing a mixture containing an excess of hydrochloric and nitrous acids to react for 5 minutes at 0°, one is assured of a complete conversion of the sulfanilic acid into the diazonium salt.

After the above reaction has occurred, an excess of sodium nitrite is added to the liquid which brings about an immediate decrease in the concentration of the hydrogen ions with the formation of sodium chloride and nitrous acid.

The work of Hantzsch,[4] Goldschmidt and his coworkers,[5] von Pechmann,[6] and many others has shown that:

1. Diazonium salts are similar to ammonium salts; hence the

[4] A summary of the elaborate researches on the subject of diazonium salts may be found in Hantzsch, A., Die Diazoverbindungen, Stuttgart, 1902.

[5] Goldschmidt, H., and Merz, A., *Ber. chem. Ges.*, 1897, xxx, 670. Goldschmidt, H., and Buss, F., *ibid.*, 1897, xxx, 2075. Goldschmidt, H., and Bürkle, E., *ibid.*, 1899, xxxii, 355. Goldschmidt, H., and Keppeler, G., *ibid.*, 1900, xxxiii, 893. Goldschmidt, H., and Keller, H., *ibid.*, 1902, xxxv, 3534.

[6] von Pechmann, H., *Ber. chem. Ges.*, 1892, xxv, 3505. von Pechmann, H., and Frobenius, L., *ibid.*, 1894, xxvii, 651.

salt of a diazonium base with a weak acid, like nitrous acid, will be hydrolyzed by water to a marked extent.

2. Diazonium hydroxides pass into diazohydrates at a rapid but measurable rate of speed.

3. The speed of coupling is directly proportional to the concentration of the diazohydrate (or its nitrosamine isomer). Thus Goldschmidt and his coworkers found that the rate of coupling of diazonium salts depended upon the degree of hydrolysis (not ionization) of the salt and not upon the concentration of the diazonium salt present. Similarly the rate of coupling of the alkali diazotates was found to be proportional to their degree of hydrolysis. Goldschmidt's work does not show, however, whether it is the diazohydrate or the nitrosamine derivative that is responsible for the coupling.

4. Compounds of unquestionable nitrosamine structure such as acetylphenylnitrosamine (nitrosoacetanilide) C_6H_5-N(NO)-$CO.CH_3$ couple readily with aromatic amines to give diazoamino compounds and with phenols to give oxyazo compounds, acetic acid being eliminated.[6] The compounds so obtained are identical with those formed with the corresponding diazonium salts.

$$C_6H_5-N(NO)-CO-CH_3 + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:N-N(H)-C_6H_4-CH_3 + CH_3-COOH$$

$$C_6H_5-N(N)-Cl + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:N-N(H)-C_6H_4-CH_3 + HCl$$

This fact has lead us to believe that it is the nitrosamine that is the active coupling agent and not the diazohydrate.

The series of changes that occur when an excess of sodium nitrite is added to a solution containing the diazonium salt of sulfanilic acid can be formulated as follows.

(2) $HO-SO_2-C_6H_4-N_2Cl + 2NaNO_2 \rightleftarrows NaO-SO_2-C_6H_4-N_2-O-N:O + NaCl + HNO_2$ (α)

(3) $NaO-SO_2-C_6H_4-N(N)-O-N:O + H_2O \rightleftarrows NaO-SO_2-C_6H_4-N(N)-OH + HNO_2$

(β)

(B) $NaO-SO_2-C_6H_4-N(N=O)-H \rightleftarrows NaO-SO_2-C_6H_4-N(HO-N)$ (A)

The finished solution, after equilibrium has been established, should contain considerable of the compounds (A) and (B) either or both of which are the active coupling agents (see formulas). We have found it necessary to allow the finished diazo reagent to stand in an ice bath for at least 15 minutes before using to get uniform results.

The above solution contains variable amounts of nitrous acid, variable because some of it escapes in the form of anhydride whenever the containing flask is opened. If a substance containing an aliphatic amino group, such as histidine, is now added to the above solution, which is the qualitative procedure usually followed, variable amounts of this amino group are replaced by the hydroxyl radical. The colors produced by the hydroxy compounds are, however, not identical with those obtained from the amino compounds and, therefore, a given amount of histidine will not always give exactly the same color under the conditions prescribed for the qualitative test, since the per cent of deamination is different in each case.

If the diazo reagent were *first* mixed with an *alkali* before adding the imidazole solution, two reactions would occur: (1) the free nitrous acid would be entirely neutralized, so there could be no disruption of the aliphatic amino groups; and (2) the equilibrium represented in Equation 3 would be displaced to the right which would lead to an increase in the concentration of the active coupling compounds (A) and (B) or to the formation of the disodium salt of (A).

$$\begin{array}{l} Na-O-SO_2-C_6H_4-N \\ \qquad\qquad\qquad\qquad\quad \| \\ Na-O-N \end{array}$$

The disodium salt represented above would be rapidly hydrolyzed by water;[5] so we would not expect it to be formed in quantity unless an excess of strong alkali, such as sodium hydroxide, were added to the solution. With a properly chosen alkali, the completed solution should contain a uniform number of active coupling molecules which should then give identical colors with the same quantity of imidazole. By a strict adherence to the directions given, which were based upon the above theoretical deductions, we have found it possible to estimate imidazole derivatives with an accuracy equivalent to that of any colorimetric method.

Sodium carbonate has been found to be the ideal alkali and it was used in preference to any other because of the following facts: (*a*) with sodium acetate no color is obtained; (*b*) sodium bicarbonate gives a color only when the bicarbonate is present in large excess (even then the color appears very slowly and is lacking in intensity); (*c*) ammonium hydroxide cannot be used because, as will be shown later, it gives a green color with diazotized sulfanilic acid; (*d*) in the presence of sodium hydroxide, weak yellow to pale orange, very labile colors are produced.

In searching for a color standard against which to match the colors produced by the reaction, it was of course natural to seek first for stable, chemically allied substances. Both methyl orange and Congo red are chemically related to the colored substances produced with the imidazoles, and we have found that Congo red alone, or mixtures of it with methyl orange do indeed give the desired colors. Combinations of these indicators ought to prove useful as color standards in other cases where the colors produced are yellow to red.

EXPERIMENTAL.

Reagents Employed.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is dissolved in 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask and water added to the mark.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Merck's or J. T. Baker's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We specify the above two grades of carbonate because they were found to give uniform results. Two other brands tried by us were found to give yellow colors that were quite different in shade from those produced with the specified salts. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumetric flask. Water is then added to the mark. This solution keeps indefinitely.

Standard Indicator Solutions.—For the estimation of *imidazole acetic, imidazole propionic acid,* and *methyl imidazole,* a solution containing 1 cc. of the stock Congo red solution in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of *histidine* or *histamine,* 1 cc. of stock Congo red and 1.1 cc. of stock methyl orange are allowed to flow into 250 cc. of water contained in a 500 cc. volumetric flask. Water is then added to the mark. The stock indicator solutions must not be mixed in concentrated form. When this is done a rapid interaction seems to occur with destruction of color.

When this standard indicator solution has been used for comparisons the symbol (CR-MO) is suffixed to the reading obtained. When this solution is properly prepared and preserved in a tightly stoppered flask, it can be kept with certainty for 2 weeks. It is best to keep this solution in a hard glass vessel, such as Pyrex, because the alkali introduced by the solution of a soft glass changes the color of the indicators enough to give untrustworthy results.

Preparation of p-Diazobenzene Sulfonic Acid Solution. (The Reagent).—1.5 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6 cc. more of the stock sodium nitrite solution are added and the well-mixed solution again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Imidazoles.

Histidine Dichloride.—This was prepared according to a slight modification of the method of Fränkel.[7] Its purity was determined by analysis after it had been dried for 48 hours in vacuum.

Ammonia was absent.

It melted with gaseous decomposition at 248°.

0.06012 gm. gave 6.52 and 6.62 cc. of nitrogen gas at 20° and 750 mm., by the Van Slyke method.

	Calculated.	Found.	
	per cent	*per cent*	*per cent*
Amino nitrogen.....	6.14	6.09	6.18

The chloride ion from 0.3009 gm. of substance was completely precipitated by 26.46 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent histidine dichloride should have used 26.40 cc.

The above two analyses prove that the histidine dichloride used was 100 per cent pure.

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[7] Fränkel, S., *Monatsh. Chem.,* 1903, xxiv, 230.

Histamine Dichloride.—The method of preparation and analysis of this compound have been previously described by us.[8]

Imidazole Propionic Acid.—Glyoxal propionic acid was prepared according to Wolff.[9] From it the imidazole propionic acid was then prepared according to Knoop and Windaus.[10] After recrystallizing the final product three times from 75 per cent acetone, too little was left to make a very exhaustive analysis. After drying in vacuum over sulfuric acid for 48 hours the pure white crystalline solid had the following properties.

It melted at 207–209°.

Ammonia was absent.

No residue on ignition.

The substance was assumed to be 100 per cent pure imidazole propionic acid.

Imidazole Acetic Acid Hydrochloride.—The substance used was obtained as a by-product in the preparation of histamine dichloride and its mode of isolation has been previously described.[11] After drying in vacuum over sulfuric acid for 48 hours the pure white, glistening solid had the following properties.

It melted at 226–228° (corrected).

Ammonia was absent.

No residue on ignition.

The chloride ion was completely precipitated from 0.1625 gm. of substance—0.10 mol—by 10.05 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent hydrochloride should have used 10.00 cc. The above properties prove that the substance used was 100 per cent pure.

4-Methyl Imidazole.—This substance was prepared from commercial glucose, ammonia, and zinc hydroxide. The directions given by Windaus and Knoop[12] were followed with the following modifications.

The solid zinc methyl imidazole hydroxide was dissolved in ten times its weight of water to which an excess of acetic acid had been added. The mixture was saturated with hydrogen sulfide under pressure and filtered from precipitated zinc sulfide. The resulting brown solution was distilled in vacuum at 50° to remove the water, hydrogen sulfide, and acetic acid. The residual dark brown gum was then extracted with ten times its weight of cold dry acetone which dissolved the methyl imidazole completely but left practically all the coloring matter behind. The oxalate was then precipitated by adding a solution of oxalic acid in acetone to the above acetone solution of methyl imidazole. The oxalate is soluble in dry acetone only in traces. The nearly white solid was then recrystallized from 75 per cent acetone until it melted at 206°. The free base was prepared

[8] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

[9] Wolff, L., *Ann. Chem.*, 1885, ccxxix, 266.

[10] Knoop, F., and Windaus, A., *Beitr. chem. Physiol. u. Path.*, 1906, vii, 144.

[11] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[12] Windaus, A., and Knoop, F., *Ber. chem. Ges.*, 1905, xxxviii, 1166.

from the oxalate by the method of Windaus and Knoop, and was obtained first as a colorless mobile oil, and then as a colorless solid which had the following properties.

It melted at 56.5°.

Ammonia was absent.

No residue on ignition.

0.2000 gm. was dissolved in 5 cc. of water and titrated with 0.1 N hydrochloric acid using dimethylaminoazobenzene as indicator. Exactly 24.40 cc. of acid were required to produce the first change in the indicator which is exactly the amount demanded for this quantity of 100 per cent methyl imidazole. The substance was, therefore, 100 per cent pure.

Procedure for Estimating Imidazoles.

The method used in developing the tables as well as the general procedure for estimating imidazoles is illustrated by the following example.

$(1-X)$ cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 seconds delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the imidazole solution are allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above. The test cylinder is then transferred to the colorimeter and set at 20 mm. The right hand cylinder which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.[13]

The speed of color formation varies with the different imidazoles as will be made clear when the individual tables are discussed. In every case, however, a maximum color intensity is reached in the course of 5 to 10 minutes and at this point the color remains stationary for a period of from 1 to 10 minutes depending upon the imidazole used. Histamine gives a promptly appearing

[13] This is the inverse of the process as it is ordinarily carried out where the standard solution is set at some definite value and the test cylinder moved until a match is obtained.

labile color whose duration of maximum intensity is about 1 minute. With histidine the color development is gradual, takes about 6 minutes to reach its maximum intensity, and lasts from 2 to 3 minutes. In every case the weaker colors are the more stable. The most accurate determinations can be obtained by choosing such an amount of imidazole solution that the standard indicator cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the imidazole-containing solution varying from 0.01 to 1.0 cc. The combined volume of water and imidazole solution used should always be 1 cc. Thus if 0.10 cc. of the imidazole solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and $1-X$ equals 0.90 cc.

Estimation of Small Amounts of Histidine.

A stock histidine solution was prepared by dissolving 2.000 gm. of 100 per cent histidine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histidine dichloride.

The color develops slowly in this case. At first a yellow color is produced which changes rapidly to orange and then more slowly to pink. A maximum color value is obtained after 5 to 6 minutes, and is maintained in the weaker solutions for about 3 minutes. Then the color changes slowly through orange to yellow.

The previously described (CR-MO) indicator was used for comparisons. The color generated by histidine contains slightly more yellow than the standard indicator solution but the difference in shade is so slight, that with practice, an intensity comparison can be made with an accuracy of from 0.5 to 3 per cent, as has been demonstrated by the determination of unknowns. Where histidine is the only substance to be determined a more perfect color match can be obtained by using more of the stock methyl orange solution in compounding the standard indicator solution. If this is done, however, Table I cannot be used. We have preferred to use this standard indicator solution because, as will be shown in the following two articles, it is more universally applicable to mixtures of imidazoles than any other.

TABLE I.

Estimation of Small Amounts of Histidine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
0.5	0.000001
1.0	0.000002
1.5	0.000003
2.0	0.000004
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030
15.5	0.000031
16.0	0.000032
16.5	0.000033
17.0	0.000034
17.5	0.000035
18.0	0.000036
18.5	0.000037
19.0	0.000038
19.5	0.000039
20.0	0.000040

TABLE I—*Concluded.*

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
20.5	0.000041
21.0	0.000042
21.5	0.000043
22.0	0.000044
22.5	0.000045
23.0	0.000046
23.5	0.000047
24.0	0.000048
24.5	0.000049
25.0	0.000050

Table I shows clearly that the color production is directly proportional to the quantity of histidine dichloride used. This fact can be conveniently represented by the formula $I \times 0.000002 = H$, where I is the depth of the indicator solution in mm. required to match the color in the test cylinder, and H is the number of gm. of histidine dichloride in the test cylinder. Thus if the indicator solution had to be set at 18.5 mm. to match the color in the test cylinder, I would equal 18.5 and H would be equal to $18.5 \times 0.000002 = 0.000037$ gm. of histidine dichloride in the test cylinder.

Estimation of Small Amounts of Histamine (β-Imidazolylethylamine).

A stock histamine solution was prepared by dissolving 2.1097 gm. of 94.8 per cent histamine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histamine dichloride.

The color develops quite rapidly in this case and becomes pink almost immediately. Although most of the color develops within 1 minute, a maximum is reached only after 4 to 5 minutes. With dilute solutions—0.00002 gm. or less—the color of maximum intensity persists for about 2 minutes. With more concentrated solutions, the period of maximum intensity may be as short as 30 seconds.

TABLE II.

Estimation of Small Amounts of Histamine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
0.8	0.000001
1.5	0.000002
2.2	0.000003
3.0	0.000004
3.7	0.000005
4.5	0.000006
5.2	0.000007
6.0	0.000008
6.7	0.000009
7.5	0.000010
8.2	0.000011
9.0	0.000012
9.7	0.000013
10.5	0.000014
11.2	0.000015
12.0	0.000016
12.7	0.000017
13.5	0.000018
14.2	0.000019
15.0	0.000020
15.7	0.000021
16.5	0.000022
17.2	0.000023
18.0	0.000024
18.7	0.000025
19.5	0.000026
20.2	0.000027
21.0	0.000028
21.7	0.000029
22.5	0.000030
23.2	0.000031
24.0	0.000032
24.7	0.000033
25.5	0.000034
26.2	0.000035
27.0	0.000036
27.7	0.000037
28.5	0.000038
29.2	0.000039
30.0	0.000040

TABLE II—*Concluded.*

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
30.7	0.000041
31.5	0.000042
32.2	0.000043
33.0	0.000044
33.7	0.000045
34.5	0.000046
35.2	0.000047
36.0	0.000048
36.7	0.000049
37.5	0.000050

The previously described (CR-MO) indicator matches the color produced by histamine perfectly.

From Table II the facts can be represented by a formula, namely,

$$I \times 0.00001333 \ldots = H_s$$

where H_s is the number of gm. of histamine in the test cylinder and I retains the meaning assigned to it under Table I.

Estimation of Small Amounts of Imidazole Propionic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole propionic acid in water, adding enough of a normal solution of hydrochloric acid to convert it into the hydrochloride, and finally diluting with water to 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of imidazole propionic acid.

A large per cent of the color develops immediately and it is red from the beginning. An accurate comparison with the indicator can be made at the end of 1 minute. Thus it was found that for 0.00001 gm. of substance a color value of 13.0 mm. (CR) was obtained at the end of 1 minute and 15.3 mm. at the end of 10 minutes which is the time required to give a maximum color at 20°. The rate of development depends to a large extent upon the temperature. Thus at 18°, 13 minutes were required to give

TABLE III.

Estimation of Small Amounts of Imidazole Propionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole propionic acid in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.5	0.000001
3.0	0.000002
4.5	0.000003
6.0	0.000004
7.5	0.000005
9.0	0.000006
10.5	0.000007
12.0	0.000008
13.5	0.000009
15.0	0.000010
16.5	0.000011
18.0	0.000012
19.5	0.000013
21.0	0.000014
22.5	0.000015
24.0	0.000016
25.5	0.000017
27.0	0.000018
28.5	0.000019
30.0	0.000020
31.5	0.000021
33.0	0.000022
34.5	0.000023
36.0	0.000024
37.5	0.000025
39.0	0.000026
40.5	0.000027
42.0	0.000028
43.5	0.000029
45.0	0.000030

a maximum of color while at 22° only 8 minutes were required. The *amount* of color finally produced is always the same regardless of the temperature. The color is unusually stable. After reaching its maximum there is no apparent change for 5 minutes.

The previously described (CR) indicator matches the color produced by imidazole propionic acid perfectly.

Table III was not extended because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can in this case be represented by the formula $I \times 0.0000006666 \ldots = P$ where P is the number of gm. of imidazole propionic acid in the test cylinder.

Estimation of Small Amounts of Imidazole Acetic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole acetic acid hydrochloride in water and diluting to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of the hydrochloride of imidazole acetic acid.

The (CR) indicator matches the color produced by imidazole acetic acid perfectly. Table IV shows the results of the test.

The statements given above for imidazole propionic acid apply equally well in this case. The facts can be represented by the formula $I \times 0.00000081308 = A$, where A is the number of gm. of imidazole acetic acid in the test cylinder.

Estimation of Small Amounts of Methyl Imidazole.

A stock solution was prepared by dissolving 2.0000 gm. of the base in 24.4 cc. of N HCl and diluting with water to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of methyl imidazole.

A large per cent of the color develops immediately which is red and comparable with the indicator from the start. The color reaches its maximum intensity in 7 minutes at 20°, and undergoes no apparent change for 15 minutes. The (CR) indicator was used for comparisons. The color match is not quite perfect in this case, as the color produced by the methyl imidazole is slightly too red for the (CR) indicator. The discrepancy in shade is so slight, however, that an accurate comparison can easily be made.

TABLE IV.

Estimation of Small Amounts of Imidazole Acetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole acetic acid hydrochloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.5	0.000002
3.7	0.000003
4.9	0.000004
6.1	0.000005
7.4	0.000006
8.6	0.000007
9.8	0.000008
11.1	0.000009
12.3	0.000010
13.5	0.000011
14.7	0.000012
16.0	0.000013
17.2	0.000014
18.5	0.000015
19.7	0.000016
20.9	0.000017
22.1	0.000018
23.4	0.000019
24.6	0.000020
25.8	0.000021
27.0	0.000022
28.3	0.000023
29.5	0.000024
30.7	0.000025
32.0	0.000026
33.2	0.000027
34.4	0.000028
35.7	0.000029
36.9	0.000030
38.1	0.000031
39.4	0.000032
40.6	0.000033
41.9	0.000034
43.0	0.000035
44.3	0.000036
45.5	0.000037
46.7	0.000038
47.9	0.000039
49.2	0.000040

Table V was not extended beyond 0.00002 gm. of methyl imidazole because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can be represented by the formula $I \times 0.00000037037 \ldots = M$ where M is the number of gm. of methyl imidazole in the test cylinder.

TABLE V.

Estimation of Small Amounts of Methyl Imidazole.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Methyl imidazole in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
2.7	0.000001
5.4	0.000002
8.1	0.000003
10.8	0.000004
13.5	0.000005
16.2	0.000006
18.9	0.000007
21.6	0.000008
24.3	0.000009
27.0	0.000010
29.7	0.000011
32.4	0.000012
35.1	0.000013
37.8	0.000014
40.5	0.000015
43.2	0.000016
45.9	0.000017
48.6	0.000018
51.3	0.000019
54.0	0.000020

Relative Value of the Standard Indicator Solutions.

The relative intensity of the two standard indicator solutions can be compared by means of the Duboscq colorimeter even though the tint is not identical. This enables one to compare readings obtained with the (CR) indicator with those obtained with the (CR-MO) indicator. The readings obtained, representing mm. on the Duboscq scale, are as follows.

(CR) standard indicator.	(CR-MO) standard indicator.
11	10
22	20
33	30

The following example will illustrate the use of the table. 0.00001 gm. of the hydrochloride of glyoxaline acetic acid has a color value on the (CR) scale of 12.3 mm. by actual determination. A calculation from the table shows that the color value on the (CR-MO) scale should be $\frac{10}{11} \times 12.3 = 11.2$ mm. The actual determination using the (CR-MO) scale, which was made fully 2 months before this comparison table was compiled, actually gave 11.2 mm. It is now possible to compare the molecular color values of the various imidazoles.

Molecular Color Value of the Five Imidazoles.

The following figures were obtained by calculating the hypothetical color value on the (CR-MO) scale that would be obtained if one molecular weight of the imidazole had been present in the test cylinder. The calculations are based on the values recorded in the tables for 0.00001 gm. of the imidazole derivatives.

	Weight. *mol*	Molecular color value.
Histidine dichloride	228.0	114,000,000
Histamine "	184.0	138,000,000
Imidazole propionic acid	140.0	191,000,000
" acetic acid as HCl	162.5	182,000,000
Methyl Imidazole	82.0	201,000,000

From this tabulation the following conclusions can be drawn.

1. The color produced by these five imidazoles depends not only upon the imidazole ring but is strongly influenced by the groups in the side chain also.

2. The color is nearly a pure red and of maximum intensity when the side chain is paraffin in character (methyl imidazole).

3. The carboxyl group makes the color more yellow and reduces its intensity. This effect is most marked when the carboxyl group is near the imidazole ring. Thus imidazole acetic acid has a molecular color value that is about 90 per cent as great as that of methyl imidazole, whereas imidazole propionic acid has a molecular color value that is 95 per cent as great as that of methyl imidazole.

4. The introduction of an amino group greatly reduces the intensity of the color and makes it decidedly yellow. When both the amino and the carboxyl group are present in the same molecule, the effect on the color is additive. Thus histamine has a molecular color value that is about 69 per cent as great as that of the methyl imidazole whereas histidine has a molecular color value that is only 57 per cent as great as that of methyl imidazole.

The Correction Blank.

When the reagent and alkali are mixed in the absence of an imidazole derivative, a very pale yellow color is produced in the course of 5 to 10 minutes that has an intensity value of 0.30 mm. (CR-MO). This same quantity of color is also produced in the presence of imidazole derivatives along with the color produced by the imidazole; so the readings obtained are too high by 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables. For example, 0.10 cc. of a histidine solution is found to have a color value equivalent to 10.3 mm. (CR-MO). The corrected reading is 10.0 mm. (CR-MO); so 0.10 cc. of this solution contains 0.00002 gm. of histidine dichloride (Table I). It is hardly necessary to say that the correction blank will be 0.30 mm. only when the test cylinder is set at 20 mm.

Substances That Do and Do Not Interfere with the Quantitative Determination of Histidine.

2 cc. of stock histidine solution were mixed in a 100 cc. precision cylinder with quantities of the other substances as stated in Table VI. Water was then added to give a final volume of 100 cc. Then 0.10 cc. of the solution was taken for the colorimetric determination.

The most active interfering substances are apparently ketones, alcohols, and ammonium salts. The volatile ketones and alcohols can always be removed by evaporation; so they offer no permanent difficulties. Amyl alcohol can be completely removed from an aqueous solution by extraction with toluene or chloroform. The resulting aqueous solution gives the correct color value. The

dissolved chloroform or toluene does not interfere with the production of color. Glycerol or glucose is seldom present, in concentrations as high as those given here, in the usual biological liquids.

TABLE VI.

Substance.	Concentration	Color.	Interference.
	per cent		
Urea.	5	Perfect.	None.
NaCl KCl Na_2SO_4 KH_2PO_4	5	"	"
Sodium acetate. Sodium citrate.	5	"	"
Glycerol.	3	"	2 per cent low in color value.
Glucose.	5	Slightly too yellow.	8 " " " " " "
Ethyl alcohol.	5 10	Perfect.	5 " " " " " " 10 " " " " " "
Methyl alcohol.	10	Slightly too yellow.	20 " " " " " "
Amyl alcohol.	Saturated aqueous solution.	" "	10 " " " " " "
Acetone.	10	Greenish yellow.	Entirely unmatchable color of slight intensity. No determination possible.
$(NH_4)_2SO_4$ free from pyridine.	1 5	Slightly brown. Greenish "	2 per cent high in color value. Entirely unmatchable color of high intensity. No determination possible.

The following substances require individual consideration.

Ammonium salts.—The positive interference referred to above for ammonium sulfate led us to believe that ammonia itself gave

a color with the reagent. Experiment proved this to be the case. A 10 per cent aqueous solution of the highest purity of ammonium sulfate obtainable, which was free from pyridine, was tested for color value by introducing 0.10 cc. of it into the test cylinder in the usual manner. A fairly intense yellowish green color developed promptly. Exactly the same quantity and quality of color were obtained when 0.50 cc. of the ammonium sulfate solution was tested.

The color value of a solution containing more than the equivalent of 1 per cent of ammonium sulfate is untrustworthy.

Proteins.—The colorimetric method is worthless when applied to a solution containing soluble proteins for two reasons. (*a*) The proteins themselves give a color with the reagent which is not quantitatively produced. Thus 0.20 cc. of a protein solution gives less than twice the color produced by 0.10 cc. (*b*) The addition of a known amount of histidine to a solution containing protein of previously determined color value gives less than the theoretical increase in color value. The results with blood proteins run about 30 per cent low.

It might be possible to eliminate this protein interference by using a stronger reagent. This would make it necessary to develop a new set of tables. No experiments have been made up to the present time to verify the correctness of the above statement.

Animal Charcoal.—We have found that animal charcoal cannot be used to decolorize a solution in which the presence of small amounts of imidazoles is to be determined, because the charcoal adsorbs appreciable quantities of these substances. Experimental data are given in Table VII to support the above statement. Kahlbaum's highest grade of powdered bone charcoal was used in these experiments. A weighed quantity of the charcoal was agitated for 10 minutes with 100 cc. of the imidazole solution. The color value of the clear solution was then determined.

Table VII shows clearly that for preparative work, or even for quantitative work where the concentration of the imidazole is high, charcoal might be used with impunity because the maximum adsorption per gm. of charcoal seems to be about 1 mg. of imidazole. It is best, however, to avoid the use of charcoal when possible.

Barium sulfate precipitates do not adsorb imidazoles.

The general method outlined will undoubtedly be found to be equally applicable to the estimation of all imidazoles with the possible exception of cyanomethyl imidazole which gives a brown and not an orange or red color. It is also applicable to the estimation of other compounds that will couple with *p*-diazobenzene sulfonic acid such as phenols and it may prove useful for the

TABLE VII.

Charcoal added.	Amount and kind of imidazole used (total volume 100 cc.).	Color value of indicator solution before adding charcoal.	Color value of indicator solution after adding charcoal.	Imidazole adsorbed by the charcoal.	
gm.	*gm.*	*mm.*	*mm.*	*gm.*	*per cent*
1	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 9.5 mm. (CR).	0.0066	66
2	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 5.5 mm. (CR).	0.0080	80
4	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 3.0 mm. (CR).	0.0090	90
1	0.02 methyl imidazole.	0.10 cc. equivalent to 54.3 mm. (CR).	0.10 cc. equivalent to 33.5 mm. (CR).	0.0075	37.5
1	0.01 histamine dichloride.	0.20 cc. equivalent to 15.3 mm. (CR-MO).	0.20 cc. equivalent to 2.0 mm. (CR-MO).	0.0090	90

estimation of aromatic amines. We hope to report the method for estimating phenols in the near future.

Our interest in these particular imidazoles will be made clear in the succeeding two papers.

SUMMARY.

1. The well known interaction between the imidazole ring and *p*-phenyldiazonium sulfonate has been made the basis of a quantitative colorimetric method for estimating histidine, histamine,

imidazole propionic acid, imidazole acetic acid, and methyl imidazole.

2. Tables are given for the direct determination of quantities of these substances ranging from 0.000001 to 0.00005 gm. The amount of imidazole in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

3. A list of substances is given, some of which do and others of which do not interfere with the determination.

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STUDIES ON PROTEINOGENOUS AMINES.

III. A QUANTITATIVE METHOD FOR SEPARATING HISTAMINE FROM HISTIDINE.

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An exact study of the metabolism of histidine has been very difficult up to the present time because we do not have the proper quantitative method for estimating imidazole derivatives. It has always been necesssary, therefore, either to isolate the imidazole derivative or to determine its presence by biological reactions. The isolation of these compounds from the usual body liquids is a very difficult task and the quantity actually isolated is probably never more than a small per cent of that actually present. The large amounts of material necessary for the isolation of imidazoles present in small quantities make the process of isolation so tedious and time-consuming that it is hardly applicable to cases where a routine analysis is desired. The biological methods, based on the action of histamine on the smooth muscle fiber system, that have been used for the identification of the base, are certainly not quantitatively exact and have not been shown to be entirely specific. A purely chemical method, applicable to small amounts of material, would be superior to any that had been previously described, if the new method were accurate and practically applicable.

In the preceding communication a method for estimating small amounts of imidazoles has been described. In that paper no claims were made for the applicability of the method to mixtures of imidazoles. It is the object of this report to show that the method can also be applied to mixtures and that the exact quantity of histamine, histidine, and methyl imidazole in such mixtures can be rapidly and accurately determined. The method has been

found to be applicable without modification to liquid media in which bacteria have been allowed to metabolize histidine in the presence of salts and glycerol or glucose.[1] In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We are trying to modify it, at the present time, so that it can be applied to such liquids. The underlying principles of the method are as follows.

The mixture of imidazole derivatives is treated with enough solid sodium hydroxide to give a 20 per cent solution of the alkali. This alkaline liquid is then extracted six times with twice its volume of amyl alcohol. This effects a separation into two fractions, the amyl alcohol extract and the alkaline aqueous liquid. The amyl alcohol extract contains all the histamine and methyl imidazole, together with ammonia and other amines if such are present in the original solution. We shall call this the *histamine fraction.* The alkaline aqueous liquid contains all the histidine, imidazole propionic, acetic, and lactic acids. We shall call this the *histidine fraction.*

The amount of histidine can then be determined by an amino nitrogen determination on the histidine fraction.

A color value determination on the histidine fraction is an index of the combined imidazole content of the liquid. If the color value determination indicates the presence of more imidazole than can be accounted for on the basis of the amino nitrogen determination, histidine is not the only imidazole present. The excess of color value can then be calculated to imidazole propionic, acetic, or lactic acids depending upon which of these substances is shown to be present by actual isolation.

Of all the imidazoles tried, methyl imidazole is the only one that is volatile with steam. It can be determined directly by collecting the distillates from a steam distillation, determining their color value, and calculating the amount of methyl imidazole from Table V of the previous article. It may be well to call attention here to the fact that imidazole itself is probably also volatile with, steam and if it is present, it will be estimated as methyl imidazole. No effort has been made to differentiate these two substances.

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

The histamine and methyl imidazole can be completely extracted from the amyl alcohol with 1.0 N H_2SO_4. A color value determination on the acid extract gives the total amount of imidazole present. By subtracting from this the color value due to the methyl imidazole, as previously determined, the color value due to histamine is obtained which can then be calculated to that substance by using Table II given in the previous paper. The bacterial experiments, described in the succeeding paper, showed methyl imidazole to be regularly absent. In this case, then, the color value determination gave the amount of histamine directly.

Sections I to VI of this report contain a detailed account of the results of experiments on known solutions of imidazole derivatives by which the accuracy of the technique of the method for separating histamine from histidine, described in Section VII, was experimentally established.

EXPERIMENTAL.[2]

I. Color Value of a Histidine-Histamine Mixture is the Sum of Their Individual Color Values.

(*a*) 1 cc. each of stock 1 per cent histidine and histamine dichloride solutions was mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.6 mm. (CR-MO).

This amount of each of the imidazoles alone would give

Histidine	5.0 mm.	(CR-MO)
Histamine	7.5 "	"
Theory	12.5 "	"

(*b*) 1 cc. of the stock histamine solution and 0.50 cc. of the stock histidine solution were mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

[2] The reagents and the terminology used are identical with those of the previous article (p. 497). The recorded readings have all been corrected by subtracting 0.30 mm., for the correction blank (p. 511).

This amount of each of the imidazoles alone would give

Histidine	2.5 mm.	(CR-MO)
Histamine	7.5 "	"
Theory	10.0 "	"

Conclusion.—Histidine and histamine do not interfere with each other's color production. Each produces its color as if the other were absent.

II. Stability of Histidine and Histamine toward Acid and Alkali.

(*a*) *Cold 20 Per Cent Sodium Hydroxide.*—1 cc. each of the stock histidine and histamine solutions was mixed with 2 gm. of solid sodium hydroxide and enough water to give a total volume of 10 cc. After standing for 1 hour at room temperature the liquid was rendered faintly acid to litmus paper with 1.0 N HCl and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.7 mm. (CR-MO).

The theory is 12.5 mm. (CR-MO) (see Section I) the result, therefore, is accurate within the limits of experimental error.

(*b*) *Hot 20 Per Cent Sodium Hydroxide.*—2 cc. of stock histidine solution were mixed with 8 cc. of water and 2 gm. of solid sodium hydroxide in a 100 cc. volumetric flask. The flask was heated on the boiling water bath for 7 hours. The cooled solution was neutralized as in (*a*) and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 8.4 mm. (CR-MO).

This, by table, is equal to 84 per cent of the histidine started with. The 7 hours heating with concentrated sodium hydroxide destroyed the imidazole ring to the extent of 16 per cent.

(*c*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*b*).

Of the final solution 0.10 cc. had a color value equivalent to 13.8 mm. (CR-MO).

This, by table, is equal to 92.5 per cent of the histamine started with. The 7 hours heating with concentrated alkali destroyed the imidazole ring to the extent of 7.5 per cent.

(*d*) *Hot Concentrated Hydrochloric Acid.*—2.00 cc. of stock histidine solution and 25 cc. of 37 per cent HCl were mixed in a 100 cc. lipped beaker and heated for 10 hours on the boiling water bath, rapid evaporation being prevented by covering the beaker with a watch-glass. At the end of the 10 hours heating the hydrochloric acid had disappeared. The dry residue was dissolved in water and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

This is the theoretical value.

(*e*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*d*).

0.10 cc. of the final solution had a color value equivalent to 15.0 mm. (CR-MO).

This is the theoretical value.

Conclusions.—Cold 20 per cent sodium hydroxide and hot concentrated hydrochloric acid do not change the color value of either histidine or histamine. Hot 20 per cent sodium hydroxide destroys 7.5 and 16 per cent of the color values of histamine and histidine respectively in the course of 7 hours. Since this loss in color value is probably caused by a destruction of the imidazole ring, it is clear that this heterocyclic ring is not perfectly stable toward hot alkali. The stability of the ring seems to depend to some extent upon the character of the side chain.

For quantitative results, a solution containing imidazoles may be evaporated to dryness with hydrochloric acid with impunity. Heating a strongly alkaline solution must be avoided.

III. Histidine Not Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

In order to prove that histidine is not extracted from a 20 per cent sodium hydroxide solution by amyl alcohol it was necessary to devise a method for extracting the alkaline solution with amyl alcohol that would be rapid and accurate. The following technique was finally adopted.

Extraction Tube and Mode of Extraction.—A 35 cc. glass-stoppered bottle was used as an extraction tube. The imidazole so-

lution, in this case 1.00 cc. of stock histidine solution, was introduced into the bottle together with enough water to give a total volume of about 10 cc. Then 2 gm. of Merck's highest purity sodium hydroxide were dissolved in the liquid, the bottle and con-

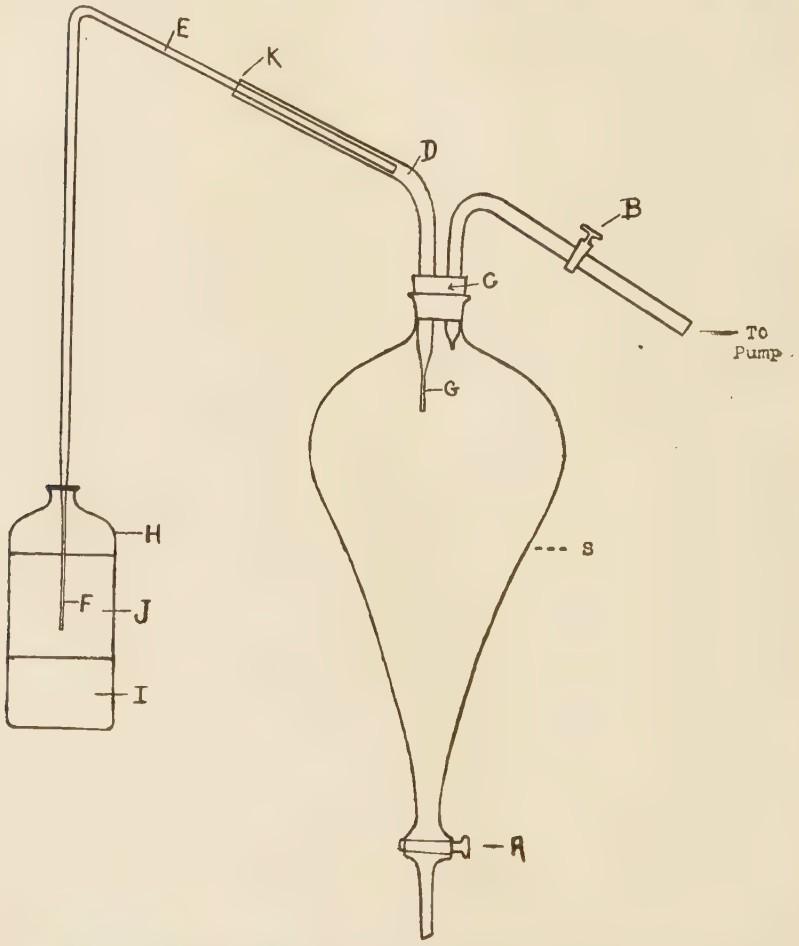

Fig. 1. A, Lower stop-cock of the Squibb separatory funnel S. B, Stop-cock to outlet tube. C, Two-hole rubber stopper. D, Wide glass tube. E, Narrow glass tube. F, Capillary tube. G, Constriction in D, about 1 mm. diameter. H, Glass-stoppered extraction tube. I, Aqueous layer. J, Amyl alcohol layer. K, A piece of rubber tubing is used at this place to connect Tubes D and E.

tents being cooled in tap water while the alkali was dissolving. 20 cc. of prepared amyl alcohol,[3] measured by graduate, were introduced into the bottle, the glass stopper[4] inserted, and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube[5] and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device shown in Fig. 1.

The 300 cc. Squibb funnel S, which must be equipped with a tightly fitting glass stopper, and the extraction tube H are supported by means of two universal clamps which in turn are attached to the same ring-stand.

After the mixture in the extraction tube has been centrifuged into two sharply defined layers, the tube is clamped into place and raised until the capillary F is immersed in the amyl alcohol layer. Stop-cock B, which should be attached to an aspirator, is then opened very slightly so that a very slight drop in pressure occurs in the Squibb funnel. The amyl alcohol is sucked through tubes E and D into the funnel. By carefully raising the extraction tube H, the amyl alcohol can be almost entirely removed without disturbing the lower layer I. The extraction tube H is then lowered, tube E swung around so that capillary F points straight up and stop-cock B opened wide to sweep the amyl alcohol from tubes D and E into the funnel.

The alkaline aqueous layer is then ready to be extracted with another 20 cc. portion of amyl alcohol. The above process was repeated five times in this case, so that a total of 100 cc. of amyl alcohol was used.

[3] The amyl alcohol was prepared by distilling Merck's highest purity product *in vacuo* and extracting the distillate once with 20 per cent sodium hydroxide solution. This gives a product that is already saturated with water and sodium hydroxide with respect to a 20 per cent sodium hydroxide solution. The volume of the aqueous alkaline layer in the extraction tube will, therefore, remain constant.

[4] The glass stopper must fit perfectly throughout its entire length. It must not be removed from the bottle until after the liquid has been centrifuged.

[5] A deep layer of cotton makes an ideal cushion for the flat bottomed bottle.

Removal of Imidazoles from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times in the same Squibb funnel with 1.0 N H_2SO_4, using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, cooled to room temperature in tap water, and diluted to exactly 100 cc. with distilled water. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 1.0 cc. had a color value equivalent to 0.20 mm. (CR-MO) which, by table, is equal to about 0.40 per cent of the histidine originally introduced.

The Alkaline Aqueous Liquid (Histidine Fraction).—The alkaline aqueous liquid was carefully transferred with water from the extraction tube to a 100 cc. precision cylinder, made slightly acid with hydrochloric acid, and, after cooling to room temperature, diluted to 100 cc. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 0.10 cc. had a color value equivalent to 5.0 mm. (CR-MO); 0.20 cc. had a color value equivalent to 10.0 mm. (CR-MO), which, by table, is equivalent to 100 per cent of the histidine introduced.

Conclusion.—Histidine is extracted from a 20 per cent sodium hydroxide solution only to the extent of about 0.50 per cent by amyl alcohol under the prescribed conditions. This is negligible in most cases.

IV. Histamine Quantitatively Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

1 cc. of stock 1 per cent histamine solution was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were

[6] The chloroform removes the amyl alcohol from the aqueous layer completely. Amyl alcohol interferes actively with the colorimetric determinations; so it is necessary to take this precaution to be sure that the readings obtained are accurate.

then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid.—The solution finally obtained had absolutely no color value beyond that of the correction blank.

Amyl Alcohol Extract (Histamine Fraction).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.5 mm. (CR-MO); 0.20 cc. had a color value equivalent to 15.0 mm. (CR-MO). This, by table, is equivalent to 100 per cent of the histamine started with.

Conclusion.—Histamine is quantitatively extracted from a 20 per cent sodium hydroxide solution by amyl alcohol.

V. Histamine Quantitatively Separated from Histidine by Means of Amyl Alcohol.

(A) When Equal Parts by Weight of Histidine and Histamine Are Present.

1 cc. each of stock histidine and histamine solutions was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted five times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid (Histidine Fraction).—Of the solution finally obtained, 0.20 cc. had a color value equivalent to 10.2 mm. (CR-MO). This is equivalent to 0.0102 gm. of histidine dichloride which is 102 per cent of the histidine originally introduced.

Amyl Alcohol Extract (Histamine Fraction).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.4 mm. (CR-MO); 0.20 cc. had a color value equivalent to 14.7 mm. (CR-MO). This is equivalent to 0.0098 gm. of histamine dichloride which is 98 per cent of the starting material.

Discussion.—In this case both results are accurate to within the limit of experimental error; but we are now certain that the low value for the histamine fraction was obtained because too few amyl alcohol extracts were made. In the following two experiments where six extracts were made instead of five, the agreement

was perfect. It is necessary, therefore, to make not less than six amyl alcohol extracts to effect a quantitative separation of histamine from histidine.

(B) When a Small Amount of Histamine Is Mixed with a Large Amount of Histidine.

5 cc. of stock histidine solution and 0.30 cc. of stock histamine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc. Of this diluted solution, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.050 gm. of histidine dichloride for the entire original solution which is a 100 per cent recovery.

Histamine Fraction.—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 22.7 mm. (CR-MO). This is equivalent to 0.003025 gm. of histamine dichloride for the entire solution which is 100.9 per cent of the starting material.

(C) When a Large Amount of Histamine is Mixed with a Small Amount of Histidine.

4.5 cc. of stock histamine solution and 0.50 cc. of stock histidine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—Of the solution finally obtained, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.005 gm. of histidine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Histamine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc.

Of this diluted solution 0.40 cc. had a color value equivalent to 13.5 mm. (CR-MO). This is equivalent to 0.045 gm. of histamine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Conclusion.—Histamine can be quantitatively separated from histidine regardless of the proportion in which each is present.

VI. Estimation of Methyl Imidazole.

(A) Methyl Imidazole Slightly Volatile with Steam from Alkaline Solution[7] *(Method A).*

1 cc. of stock 1 per cent methyl imidazole was diluted to 100 cc. in a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated on the boiling water bath until the final volume of the alkaline liquid was about 5 cc. An excess of hydrochloric acid was then carefully added and the solution evaporated until all the water and acid had volatilized. The residue was transferred, with water, to a 100 cc. volumetric flask and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 20.2 mm. (CR), which is equivalent to 0.0075 gm. of methyl imidazole or 75 per cent of the amount originally introduced. This treatment will volatilize 0.0025 gm. of methyl imidazole; so if less than this amount is present in the solution it should be determinable by this method which we shall call Method A. The mode of applying this method to a practical unknown is given in Section VII.

(B) Methyl Imidazole Volatilizes Slightly from a Solution Containing Hydrochloric Acid.[7]

1 cc. of stock methyl imidazole was diluted with water to about 100 cc. in a glass dish. Then 5 cc. of 37 per cent HCl were added and the solution evaporated on the water bath until all the water and acid had been volatilized. The residue was transferred to a volumetric flask and diluted with water to 100 cc.

[7] These two experiments, (*A*) and (*B*), do not prove that the methyl imidazole was actually lost by volatilization. That this imidazole derivative is really volatile with steam was conclusively demonstrated and the proof of this statement is given under Method B.

Of the solution so obtained 0.10 cc. had a color value equivalent to 23.7 mm. (CR), which is equivalent to 0.00875 gm. of methyl imidazole or 87.5 per cent of the amount originally introduced. It appears, then, that 0.00125 gm. of methyl imidazole volatilized even in the presence of hydrochloric acid. This may mean either that methyl imidazole hydrochloride is hydrolyzed by water and that the base formed by hydrolysis is volatilized, or that methyl imidazole hydrochloride is itself volatile. The following experiment has convinced us that it is the hydrochloride that is volatile.

These experiments are of value because they show clearly that a solution in which methyl imidazole is to be determined must not have been evaporated in the presence of either alkali or hydrochloric acid.

(C) Methyl Imidazole Will Not Volatilize from a Solution Containing Sulfuric Acid.

1 cc. of stock methyl imidazole solution was diluted with 100 cc. of water in a glass dish. Then 1 cc. of 95 per cent H_2SO_4 was added to the solution and the liquid evaporated to a final volume of about 5 cc. This was transferred to a volumetric flask, neutralized to litmus paper, and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 27.0 mm. (CR), which is equivalent to 100 per cent of the amount introduced.

When it is necessary to concentrate a solution before determining its color value, this can be safely done after adding an excess of sulfuric acid.

(D) Direct Determination of Methyl Imidazole (Method B).

5 gm. of anhydrous sodium carbonate, 5 cc. of water, and 1 cc. of stock methyl imidazole solution were subjected to a steam distillation from a 200 cc. distilling flask, the volume of the methyl imidazole-containing solution being kept constantly at from 5 to 10 cc.[8] The distillate was collected in a 250 cc. graduated precision cylinder.

[8] The liquid from which the methyl imidazole is to be volatilized must be kept saturated with sodium carbonate. Methyl imidazole is practically non-volatile from a dilute sodium carbonate solution.

Collections.—The volume of the first distillate was 154 cc., of which 0.10 cc. had a color value equivalent to 14.8 mm. (CR). The entire 154 cc. contained 0.00847 gm. of methyl imidazole.

The volume of the second distillate was 153 cc. of which 0.50 cc. had a color value equivalent to 8.7 mm. (CR). The entire 153 cc. contained 0.0009945 gm. of methyl imidazole.

The volume of the third distillate was 150 cc. of which 1.0 cc. had a color value equivalent to 6.0 mm. (CR). The entire 150 cc. contained 0.000338 gm. of methyl imidazole.

The volume of the fourth distillate was 140 cc. of which 1.0 cc. had a color value equivalent to 1.10 mm. (CR). The entire 140 cc. contained 0.00007 gm. of methyl imidazole.

There was thus accounted for 0.0098725 gm. of methyl imidazole which is 98.7 per cent of the amount originally introduced.

(*E*) *Proof That Methyl Imidazole Can Be Estimated by Method B in the Presence of Other Imidazoles.*

1 cc. each of the stock histidine, histamine, methyl imidazole, imidazole propionic acid, and imidazole acetic acid solutions was mixed and subjected to steam distillation under conditions identical with those given above. Six distillates were collected, the combined content of which was 0.009831 gm. of methyl imidazole which is 98.31 per cent of the methyl imidazole originally introduced. This proves conclusively that of the five imidazoles tried, only methyl imidazole is volatile with steam, and that it can be separated from other imidazoles by this method. Since methyl imidazole is chemically similar to *p*-cresol and scatole, both of which have been found in animal excretions, it will be of interest to see if methyl imidazole cannot also be found in such excretions. We have been unable to establish this as a fact up to the present time.

(*F*) *Method C for Estimating Methyl Imidazole.*

Method A has proved to be untrustworthy when applied to solutions containing the bacterial decomposition products of glycerol because evaporation with alkali gives rise to non-volatile substances that interfere with the colorimetric determinations. The amyl alcohol extract from such solutions is free from these decomposition

products. It should be possible, therefore, to determine methyl imidazole by evaporating the *histamine fraction* with alkali as described under Method A, instead of the *original solution.* In this case a color value determination on the histamine fraction would give the total amount of imidazole present. The solution would then be evaporated to dryness with 5 cc. of M Na_2CO_3 solution, the residue dissolved in hydrochloric acid and the solution reevaporated. The residue would be dissolved in water and diluted to the original volume of the histamine fraction. The color value so obtained would be *lower* than that of the original histamine fraction only when methyl imidazole was originally present and the decrease in color value could easily be calculated to methyl imidazole. For bacterial metabolism studies this method has been found to be quite accurate and shorter than Method B. Its application is more specifically given in Section VII.

VII. Separation of Histamine and Methyl Imidazole from Histidine, Imidazole Propionic Acid, and Imidazole Acetic Acid. The Accurate Determination of Histamine, Methyl Imidazole, and Histidine.

The method outlined below is a duplicate of the method that was used in the bacterial metabolism studies to be described in the following paper. Some of the steps were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions: 5 cc. of 2 per cent histidine dichloride, 4 cc. of 1 per cent histamine dichloride, 2 cc. of 1 per cent imidazole propionic acid, 2 cc. of 1 per cent imidazole acetic acid, and 2 cc. of 1 per cent methyl imidazole. This was mixed with 10 cc. of 2 per cent ammonium chloride, 4 cc. of glycerol, 100 cc. of Salt Medium 1[1], and 76 cc. of water in a 300 cc. Pyrex Florence flask.

Hydrogen Ion Concentration.—1 cc. of the finished solution was treated with 0.10 cc. of a 0.01 per cent solution of phenolsulfonephthalein and the color produced compared with a set of standard tubes.[9] In this way the original solution was found to have a pH of 6.8.

[9] Furnished by Hynson, Westcott, and Dunning, Baltimore, Md.

Filtration.—The remaining solution was forced through a Berkefeld filter. The flask and filter were washed free from imidazoles with 200 cc. of distilled water. The filtrate was treated with 1 cc. of 95 per cent sulfuric acid, transferred to a 200 cc. glass dish and evaporated on the water bath until salts began to crystallize. The residue was carefully transferred to a graduated precision cylinder with enough water to give a final volume of exactly 25 cc. This will be referred to as the test solution.

Total Color Value.—2.50 cc. of the test solution were diluted to 100 cc. in a volumetric flask. Of this solution 0.10 cc. had a color value equivalent to 17.7–17.9 mm. (CR-MO) the color being slightly too red for the standard indicator. 0.10 cc. of this solution should contain

	gm.		*mm.*	
Histidine dichloride	0.00001	Color value	5.0	(CR-MO)
Histamine "	0.000004	" "	3.0	"
Methyl imidazole	0.000002	" "	4.9	"
Imidazole propionic acid	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory			17.9	"

The check between fact and theory is perfect which adds weight to the statement made in Section I. That so close a check should have been obtained is surprising when the different rates of color development for these five substances are considered. The results obtained here show that *imidazoles are not adsorbed by a Berkefeld filter.*

Methyl Imidazole (*Method A*).—The remaining 98 cc. of the above solution were transferred to a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated to a volume of about 5 cc. 50 cc. of water were then added and the solution reevaporated to a volume of 5 cc. Finally 5 cc. of 37 per cent HCl were added carefully and the liquid was evaporated until all the water and acid had volatilized. The residue was dissolved in water and diluted to exactly 98 cc.

Of this solution 0.10 cc. had a color value equivalent to 13.2 mm. (CR-MO). $17.9 - 13.2 = 4.7$ mm. (CR-MO) or 5.2 mm. (CR)[10] was the color value due to methyl imidazole. This, by

[10] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

table, is equivalent to 0.0193 gm. of methyl imidazole for the entire test solution which is 97 per cent of that actually introduced.

Separation of the Histamine from the Histidine Fraction.—10 cc. of the test solution were measured into the extraction tube. 3 gm. of solid sodium hydroxide were added to the solution and the mixture cooled in tap water while the alkali was dissolving. The strongly alkaline aqueous liquid was extracted six times with prepared amyl alcohol in the manner described in Section III.

Histidine Fraction.—The alkaline liquid was carefully transferred to a graduated precision cylinder with 10 cc. of 20 per cent N HCl. The liquid, which was still alkaline to litmus paper, was rendered faintly acid by adding 95 per cent H_2SO_4 drop by drop. Water was then added to the cooled solution to bring its volume up to exactly 25 cc. About 10 cc. of chloroform were finally added[6] and the mixture was vigorously agitated.

Color Value.—6.25 cc. of the clear aqueous layer were diluted to 100 cc. in a precision cylinder and vigorously agitated with about 20 cc. of chloroform. Of the clear aqueous layer, 0.10 cc. had a color value equivalent to 11.0 mm. (CR). In this case the (CR) indicator gave a better match than the (CR-MO) indicator. 11.0 mm. (CR) are equivalent to 10.0 mm. (CR-MO).[10] The color value of this solution should have been

	gm.		*mm.*	
Histidine dichloride	0.00001	Color value	5.0	(CR-MO)
Imidazole propionic acid	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory			10.0	"

The agreement was perfect. This proves that histidine, imidazole propionic acid, and imidazole acetic acid are quantitatively retained by the alkali and appear together in the histidine fraction. Of these three substances only one, histidine, can be determined directly. We have been unable to devise a method for estimating either imidazole acetic or propionic acid separately, so that if both should appear in an unknown it would be impossible to differentiate between them. Their combined quantity can be determined by subtracting from the total that part of the color value that is due to histidine, as calculated from the Van Slyke determination, and calculating the excess color value to either imidazole propionic or acetic acid.

Amino Nitrogen Determination (Histidine).—The amino nitrogen in 5 cc. of the histidine fraction was determined by the Van Slyke method using the micro-apparatus. There was thus obtained 0.88 cc. of N_2 at 16° and 749 mm., which is equivalent to 0.1024 gm. of histidine dichloride for the entire test solution. This is 102.4 per cent of the amount originally introduced which is within the limit of experimental error for so small a quantity of substance. This proves that the ammonia present in the original test solution has passed into the amyl alcohol extract so that no special precautions need be taken to free the histidine fraction from ammonia. This statement has been amply verified by experiments described in the following paper. It need hardly be said that this method will be an accurate index of the quantity of histidine in the test solution only when other amino-acids are absent.

The Histamine Fraction.—The combined amyl alcohol extracts were extracted with 1.0 N H_2SO_4 as has already been described under Section III. The solution finally obtained was too concentrated to make an accurate determination possible, so 12.5 cc. of it were diluted to 50 cc. in a volumetric flask.

Of this diluted solution 0.10 cc. had a color value equivalent to 8.7 mm. (CR) or 7.9 mm. (CR-MO) and 0.20 cc. had a color value equivalent to 17.4 mm. (CR) or 15.8 mm. (CR-MO).

If the methyl imidazole and histamine had been entirely extracted by the amyl alcohol, 0.10 cc. of this solution should have had a color value equivalent to 7.9 (CR-MO). The check is perfect.

Removal of Ammonia and Methyl Imidazole by Evaporation with Alkali.—The entire histamine fraction was recombined and evaporated in a glass dish with 5 cc. of a M Na_2CO_3 solution until a dry residue was obtained. This was then dissolved in water, treated with an excess of 37 per cent HCl, and the resulting solution again evaporated to remove the water and acid entirely. The solid so obtained was transferred to a precision cylinder with enough water to give a final volume of 20 cc.

Amino Nitrogen Determination (Histamine).—Of the above solution, 5 cc. gave 0.56 cc. of N_2 at 25° and 747 mm., which is equivalent to 0.04015 gm. of histamine dichloride for the entire test solution and is 100.4 per cent of that actually introduced. Although this method works ideally when pure substances are

used, it occasionally gives high results when applied to bacterial metabolism experiments because this fraction sometimes contains other non-volatile amines.

Color Value Determination (Methyl Imidazole by Method C).—5 cc. of the concentrated histamine solution were diluted to 100 cc. in a volumetric flask. Of this diluted solution 0.20 cc. had a color value equivalent to 6.0 mm. (CR-MO) which is equivalent to 0.04 gm. of histamine dichloride for the entire test solution and 100 per cent of the amount originally introduced. The evaporation with the alkali produced a loss in color value of 15.8 − 6.0 = 9.8 mm. (CR-MO) or 10.8 mm. (CR) for 0.20 cc. of the solution which, by table, is equivalent to 0.02 gm. of methyl imidazole for the entire test solution and is 100 per cent of the amount originally introduced.

This method gives the exact quantities of both histamine and methyl imidazole. As will be shown in the following paper, equally exact values are obtained when bacterial metabolism liquids are examined.

Determination of Ammonia.

The method of Van Slyke and Cullen[11] was used for the determination of ammonia. 0.10 N HCl and NaOH were used in place of the 0.02 N acid and alkali recommended by the above authors. 5 gm. of solid potassium carbonate were added to 5 cc. of the test solution and the mixture aerated for 1 hour. The liberated ammonia neutralized 7.25 cc. of the 0.1 N acid as compared to 7.28 cc. demanded by the theory for 97.4 per cent ammonium chloride which was the purity of the salt originally introduced.

CONCLUSION.

A method has been devised for separating histamine and methyl imidazole from histidine, and imidazole propionic and imidazole acetic acids.

To Professor F. C. Koch, acting Chairman of the Department of Physiological Chemistry, we are indebted for suggestions and advice, in various ways, during the course of this work. We wish, herewith, to acknowledge his aid and to express our thanks for his many kindnesses.

[11] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 218.

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STUDIES ON PROTEINOGENOUS AMINES.

IV. THE PRODUCTION OF HISTAMINE FROM HISTIDINE BY BACILLUS COLI COMMUNIS.

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INTRODUCTION.

β-imidazolylethylamine (4-β-aminoethylglyoxaline), or histamine as it is appropriately called, was first obtained by Windaus and Vogt[1] in 1907 through synthesis from imidazole propionic acid. As a product of putrefaction of histidine it was first isolated by Ackermann[2] in 1910, who inoculated a synthetic medium containing histidine hydrochloride, peptone, glucose, and the necessary mineral salts with a piece of beef pancreas which had been allowed to putrefy for 24 hours. Aside from histamine, Ackermann was also able to separate a small quantity of imidazole propionic acid from the putrefaction mixture. In the same year the remarkale physiological properties of histamine were discovered independently by Barger and Dale,[3] and by Kutscher,[4] who found that it was one of the active principles of ergot. The physiological action of the base was then studied in great detail by many investigators, especially by Dale[5] and his coworkers. In 1911 Barger and Dale[6] obtained histamine from the mucosa of the small intestine of the ox and ascribed the depressor action of secretin, at least in part, to this base. The question whether histamine is a normal product of the intestinal mucosa or whether it is found there as a product of the bacterial decomposition of proteins containing histidine was decided in favor of the latter assumption by Mellanby and Twort[7] who isolated, from

[1] Windaus, A., and Vogt, W., *Ber. chem. Ges.*, 1907, xl, 3691.

[2] Ackermann, D., *Z. physiol.. Chem.*, 1910, lxv, 504.

[3] Barger, G., and Dale, H. H., *Proc. Physiol. Soc.*, 1910, xxvi, 128; *Zentr. Physiol.*, 1910–11, xxiv, 885; *J. Chem. Soc.*, 1910–11, xcvii, 2592.

[4] Kutscher, F., *Centr. Physiol.*, 1910–11, xxiv, 163.

[5] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910–11, xli, 318; 1911–12, xliii, 182.

[6] Barger, G., and Dale, H. H.; *J. Physiol.*, 1910–11, xli, 499.

[7] Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912–13, xlv, 53.

the intestinal contents of the guinea pig, a bacillus of the colon-typhoid group capable of converting histidine into histamine. In the same year Berthelot and Bertrand[8] isolated a bacillus from the human intestine that resembled Friedländer's pneumobacillus in all its morphological and chemical characteristics, but which had the additional faculty of decarboxylating histidine to histamine. They named this organism *Bacillus aminophilus intestinalis*.

This apparently simple chemical process, the decarboxylation of amino-acids to exceedingly potent substances of amine structure, is of great theoretical and practical interest. The relation of this problem to the general nutrition of bacteria, to the metabolism of amino-acids in the mammalian organism, to the pathology and pharmacology of the smooth muscle fiber system, and to the chemical constitution of the products of the glands of internal secretion, mark it as a fundamental inquiry of biology. The systematic investigation of these problems has been under way in this laboratory for the past 4 years.

Theoretical considerations and preliminary experiments had convinced us that the ability to decarboxylate amino-acids to amines could not be restricted to one or two species of bacteria but might well be a fairly common property of microorganisms. It soon became apparent that to attack the problem successfully it would be necessary to devise quantitative chemical methods for the determination and separation of the various proteinogenous amines as well as the other possible products resulting from the catabolism of the amino-acids. The preparation of the amino-acids, amines, intermediates, and by-products was an obvious preliminary to the attack on the bacterial metabolism experiments. We have already reported in detail on the preparation of β-imidazolylethylamine.[9] The preceding two articles in this *Journal* describe the quantitative methods used for the separation and estimation of imidazoles. It is the object of this communication to show what the optimum conditions are for the conversion of histidine to histamine by the colon bacillus. It is our intention to report a series of experiments in the near future that will show which of the common pathogenic and non-patho-

[8] Berthelot, A., and Bertrand, D.-M., *Compt. rend. Acad.*, 1912, cliv, 1643, 1826.

[9] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

genic bacteria possesses the faculty of decarboxylating histidine. Work is also under way to apply similar methods to a study of the decarboxylation of tyrosine and tryptophane.

EXPERIMENTAL.

Foreword.

We have divided the experimental material presented in this article into three parts.

Part I contains a description of seventeen experiments that show the effect of *the composition of the medium* upon the metabolism of histidine by the colon bacillus.

The experiments described in Part II were carried out to ascertain *how the amount of histamine produced varied with the duration of the experiment.*

Part III contains a description of four experiments that seem to throw some light on *the causal relation between the formation of histamine and the hydrogen ion concentration of the medium.*

Part I. Effect of the Composition of the Medium upon the Metabolism of Histidine by the Colon Bacillus.

Preparation of Stock Solutions.

Stock Histidine Solution.—Vacuum-dried, 100 per cent histidine dichloride—4.0000 gm.—was dissolved in water and diluted to exactly 200 cc. in a volumetric flask.

Stock Ammonium Chloride Solution.—J. T. Baker's 97.4 per cent ammonium chloride—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Stock Potassium Nitrate Solution.—Kahlbaum's 100 per cent potassium nitrate—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Salt Medium 1.—0.30 gm. of potassium chloride.
8.00 " of potassium dihydrogen phosphate.
24.00 " of sodium chloride.
0.40 " " sulfate (anhydrous).
8.00 " " bicarbonate.
0.20 " calcium chloride (anhydrous).

These amounts were dissolved in water and diluted to exactly 2,000 cc. When properly prepared the salt medium should contain no precipitate.

Mode of Preparing Completed Media.

The most complex media were Nos. 15 and 16. They were prepared as follows:

10 cc. each of the stock histidine and ammonium chloride solutions and 5 cc. of stock potassium nitrate solution were measured, by means of normal pipettes, into a 300 cc. Pyrex Florence flask. 4 cc. of glycerol, measured by graduate, were washed into the Pyrex flask with 71 cc. of water. Exactly 100 cc. of salt medium were then added.

Each of the seventeen completed media had a final volume of 200 cc. before sterilization, and contained exactly 0.2000 gm. of histidine dichloride.

Nos. 1 and 2 contained 10 cc. of stock 2 per cent histidine dichloride,
100 " " salt medium,
90 " " water.

Nos. 3 and 4 contained 10 cc. of stock 2 per cent histidine dichloride,
10 " " " 2 " " ammonium choride,
100 " " salt medium,
80 " " water.

Nos. 5 and 6 contained 10 cc. of stock 2 per cent histidine dichloride,
4 " " glycerol,
100 " " salt medium,
86 " " water.

Nos. 7 and 8 contained 10 cc. of stock 2 per cent histidine dichloride,
10 " " " 2 " " ammonium chloride,
4 " " glycerol,
100 " " salt medium,
76 " " water.

Nos. 9 and 10 contained 10 " " stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
100 " " salt medium,
85 " " water.

Nos. 11 and 12 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
10 " " " 2 " " ammonium chloride,
100 " " salt medium,
75 " " water.

Nos. 13 and 14 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
4 " " glycerol,
100 " " salt medium,
81 " " water.

Nos. 15 and 16 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate
10 " " " 2 " " ammonium chloride,
4 " " glycerol,
100 " " salt medium,
71 " " water.

No. 17 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
10 " " " 2 " " ammonium chloride,
5 gm. of crystalline glucose,
100 cc. of Salt Medium 1,
70 " " distilled water.

Sterilization.

The seventeen flasks were stoppered with cotton and sterilized by autoclaving for 1 hour at 10 pounds pressure. Calcium phosphate precipitated as carbon dioxide escaped.

Hydrogen Ion Concentration before Inoculation.

This was determined by the method described on page 534. Each of the sixteen completed media had a pH of 7.35 before inoculation.

Inoculation with Bacillus coli communis.

The culture used was a laboratory stock culture, originally isolated from human feces.

The 24 hours growth on sixteen agar slants was transferred with sterile salt solution to a sterile 25 cc. graduated precision cylinder. The emulsion was then diluted to 25 cc. with sterile salt solution.

Method for Counting Bacteria.

Of the well mixed emulsion, 1 cc. was diluted to 250 cc. Of this dilute suspension, 0.01 cc. was spread out smoothly over an area of 10 sq. cm. and the bacteria stained with 5 per cent aqueous fuchsin. They were then counted by the method of Breed and Brew[10] which showed that about 9,000,000,000 bacteria were

[10] Breed, R. S., and Brew, J. D., *New York Agric. Exp. Sta., Tech. Bull. 49,* 1916.

present in every cc. of the original bacterial emulsion. Exactly 1 cc. of the original emulsion was transferred to each of the sixteen flasks.

Incubation.

The nine odd numbered flasks were placed in an incubator. This is the aerobic series. The eight even numbered flasks were placed in a large vacuum desiccator with a dish containing pyrogallic acid and sodium hydroxide. The desiccator was completely exhausted, placed in the incubator, and reexhausted once a day to insure against leakage which was very slight. This is the anaerobic series.

The mixtures were incubated for 14 days.

Growth of Bacteria.

Judging from the degree of turbidity of the solutions, the bacteria grew best in Flasks 7, 8, 13, 14, 15, and 16. A very good growth was also obtained in Flasks 5, 6, 11, and 17. Flasks 12, 9, 10, 3, 4, 1, and 2 had the least growth in descending order.

The aerobic solutions were pale yellow to green, the anaerobic were colorless to very pale yellow.

Filtration.

The contents of each flask were forced through a clean 2.5 inch Berkefeld filter. The flask and filter were then washed free from imidazoles with about 200 cc. of distilled water. The filtrate obtained was always perfectly clear.

Hydrogen Ion Concentration after Incubation and Filtration.

1 cc. of the undiluted filtrate was used for the pH determination. The following results were obtained.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
8.3	7.6	8.2	7.4	7.15	7.45	>6.6	>6.6	8.4	8.0	8.3	7.9	>6.6	>6.6	>6.6	>6.6	>6.6

Test Solution.

The filtrate and washings were treated with 1 cc. of 95 per cent H_2SO_4 and evaporated in a 200 cc. glass dish until salts began to crystallize. This residue was then transferred to a precision cylinder with enough water to give a final volume of 25 cc. This liquid will be referred to as the test solution.

Total Color Value.

2.50 cc. of the test solution were diluted to exactly 100 cc. Portions of this diluted solution were then used for the colorimetric determinations. The results obtained are given in Table I.

TABLE I.
Total Color Value.

Flask No.	Diluted solution.	Color value of (CR-MO) indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
1	1.0	2.0	Brown, match poor.	2.0
2	0.10	6.6		66.0
	0.20	13.2		
3	1.0	3.5	Brown, match poor.	3.5
4	0.10	10.1		98.0
	0.20	20.3		
5	0.10	9.0		90.0
	0.20	18.0		
6	0.10	10.4		104.0
	0.20	20.8		
7	0.10	9.4		94.0
	0.20	18.8		
8	0.10	10.0		100.0
	0.20	20.0		
9	1.0	3.7	Brown.	3.7

TABLE I.—*Concluded.*

Flask No.	Diluted solution.	Color value of (CR-MO) indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
10	0.10	9.1		91.0
	0.20	18.2		
11	1.0	2.7	Brown.	2.7
12	0.10	10.0		100.0
	0.20	20.0		
13	0.10	10.0		100.0
	0.20	20.0		
14	0.10	9.7		97.0
	0.20	19.4		
15	0.10	10.0		100.0
	0.20	20.0		
16	0.10	10.0		100.0
	0.20	20.0		
17	0.10	9.3		93.0
	0.20	16.0		

Separation of the Histamine from the Histidine Fraction.

This separation was conducted on 10 cc. of the test solution exactly as has already been described on page 534. No separation was conducted on Flasks 1, 3, 9, and 11, because the total color value determination showed that the imidazole ring had been almost completely destroyed in these cases.

Histidine Fraction.—Of the histidine fraction, whose final volume was 25 cc., 6.25 cc. were diluted to 100 cc. for the color value determination, and 5 cc. portions were analyzed for amino nitrogen, all the steps being duplicates of the process already described. The results are given in Table II.

TABLE II.

Histidine Fraction.

Flask No.	Solution used.	Color value of (CR-MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
2	0.10 0.20	6.4 12.7	64	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. **64.5** %	0
4	0.10 0.20	10.1 19.8	98	1.80 cc. N_2 at 22.5° and 741 mm. 0.2008 gm. histidine Cl_2. **100.4** %	0
5	0.10 0.20	7.9 15.8	79	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. **81.5** %	0
6	0.10 0.20	7.4 14.8	74	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. **58.1** %	8.15
7	0.10 0.20	4.6 9.2	46	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. **46.0** %	0
8	0.10 0.20	6.4 12.7	64	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. **65.5** %	0
10	0.10 0.20	8.9 17.8	89	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. **89.6** %	0
12	0.10 0.20	9.8 19.7	98	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. **100.0** %	0
13	0.10 0.20	4.0 8.0	40	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. **39.5** %	0

TABLE II—*Concluded.*

Flask No.	Solution used.	Color value (CR-MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
14	0.10 0.20	9.0 18.1	90	1.60 cc. N_2 at 23° and 753 mm. 0.1807 gm. histidine Cl_2. **90.4 %**	0
15	0.10 0.20	4.5 9.0	45	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0 %**	0
16	0.10 0.20	7.5 15.0	75	1.35 cc. N_2 at 20° and 753 mm. 0.1549 gm. histidine Cl_2. **77.4 %**	0
17	0.10 0.20	5.3 8.2	53	1.06 cc. N_2 at 24° and 737 mm. 0.1168 gm. histidine Cl_2. **58.4 %**	0

Discussion.—The colors produced by the histidine fraction were indistinguishable from those produced by pure histidine excepting No. 6. In this case the color produced was slightly too pink for the indicator which would suggest the presence of imidazole propionic, acetic, or lactic acid. The color value of No. 6 was equivalent to 74 per cent of the original color value. The Van Slyke determination showed that only 58 per cent of the histidine originally introduced was still present. It was quite clear, therefore, that in this one case a part of the histidine had been deaminized. We did not determine the character of the product but assumed it to be imidazole propionic acid because this substance has actually been isolated by Ackermann[2] as a product of the bacterial metabolism of histidine. If 58 per cent of the original color value is assumed to be due to histidine, this would be equivalent to 5.8 mm. (CR-MO) for 0.10 cc. of the solution. The difference between this and the observed color value—1.6 mm. (CR-MO)—would be due to imidazole propionic acid. This is equivalent to 0.01 gm. of imidazole propionic acid for the entire test solution

which shows that 8.15 per cent of the histidine was converted into this substance.

We wish to call attention here, to the close check between the histidine values obtained by the Van Slyke and the colorimetric methods. The entire significance of this agreement will be discussed in detail at the end of the article. This agreement was not so close in No. 17 where glucose was used in place of glycerol. The products formed by the metabolism of glucose interfere with the color production. This is proved quite clearly by the discrepancy between the color values obtained from 0.20 and 0.10 cc. of the solution. Since 0.10 cc. of the solution had a color value equivalent to 5.3 mm. (CR-MO), 0.20 cc. should have had a color value equivalent to 10.6 mm. The value actually obtained was 8.2 mm. which shows that the color production had been seriously interfered with. A smaller interference would be expected from 0.10 cc. because less of the objectionable substances were introduced. For that reason the calculations were based upon the color value for 0.10 cc.

Histamine Fraction.—This was obtained by extracting the combined amyl alcohol extracts with 1.0 N H_2SO_4 as has already been described under Sections III and VII of the previous paper. The final volume of the neutral solution was always 100 cc. The colorimetric determinations were made on this solution without further dilution. That portion of the histamine fraction which was not used for the colorimetric determinations—99 cc. or more—was treated with 5 cc. of M Na_2CO_3 solution and evaporated to dryness on the water bath. An excess of 37 per cent HCl was then added and the liquid reevaporated to remove the water and acid entirely. The residue was transferred to a graduated precision cylinder with enough water to give a final volume of 25 cc. This concentrated solution, which was now free from ammonia and volatile amines, was used for the following determinations.

1. An Amino Nitrogen Determination.—This was carried out in only a few cases because the presence of about 10 per cent of histamine was regularly indicated by the Van Slyke method when the colorimetric determination showed this substance to be entirely absent. We have found more recently (see Sections II and III of Paper III) that the Van Slyke method can be used for the estimation of histamine when that substance is produced in large

amounts. The entire significance of these facts will be discussed in detail at the end of this section.

2. A Color Value Determination.—In case this determination agreed with the previous determination it served as a check, or if it had been lower than the previous determination it would have *indicated* the presence of methyl imidazole. The redetermined color value always agreed perfectly with that previously determined, which proved that methyl imidazole was regularly absent.

3. A biological verification of the presence of histamine.

4. A chemical verification of the presence of histamine.

The results are given in Table III.

TABLE III.

Histamine Fraction.

Flask No.	Amount of solution used.	Color value of (CR-MO) indicator.	Histamine.		Histidine converted into methyl imidazole by Methods B and C.
			In test solution.	Histidine converted.	
	cc.	*mm.*	*gm.*	*per cent*	*per cent*
2	1.0	3.0 Brown.	0.0009	0.45	None.
4	1.0	4.0 Too yellow.	0.00125	0.6	"
5	1.0	4.0 " "	0.00125	0.6	"
6	1.0	3.8 " "	0.0012	0.6	"
7	0.05	11.2	0.075	46.4	"
	0.10	22.2			
8	0.10	6.6	0.0221	13.7	"
	0.20	13.1			
10	1.0	3.8 Too yellow.	0.0012	0.6	"
12	1.0	4.0 " "	0.00125	0.6	"
13	0.05	12.4	0.0825	51.0	"
	0.10	24.7			
14	0.20	6.4	0.01075	6.7	"
	0.40	12.8			
15	0.05	12.1	0.0807	50.0	"
	0.10	24.2			
16	0.10	7.5	0.025	15.5	"
	0.20	15.0			
17	0.10	12.6	0.0425	26.3	"
	0.20	25.3			

Discussion.—The color value of the histamine fraction never was nil. Values lower than 4.0 mm. (CR-MO) for 1.0 cc. of the solution were due to *histidine* not *histamine* (see p. 525). The constancy of this value in those cases where histamine was absent is striking. The color development for histamine is characteristic of that substance and is hard to confuse with that of either histidine or methyl imidazole. In those seven cases where histamine is recorded as present, the color was identical with that obtained from pure histamine. Further proof of the correctness of this method as a means of determining histamine is given below.

Biological Verification of the Presence of Histamine.

A portion of the solution, which had been shown to contain 1 mg. of histamine dichloride by the colorimetric method, was injected into the femoral vein of a dog that had been previously anesthetized and prepared so that a blood pressure and respiratory tracing could be obtained at the same time. Figs. 1 and 2 are representative tracings. Similar tracings were obtained from Samples 7, 8, 13, 14, and 17. The entire Solution 5, which can be taken as a representative of those solutions in which a color value of 4.0 mm. (CR-MO) per 1 cc. was obtained, was injected. There was no apparent change either in blood pressure or respiration. The curve for No. 15 shows an effect that is usually not obtained. The injection was started and interrupted after a fraction of a drop of the solution had entered the circulation. Although this small quantity of substance produced a marked bronchial spasm, it raised the blood pressure very slightly. The subsequent injection of the remainder of the mg. of substance produced the customary drop in blood pressure, together with an intense bronchial spasm.

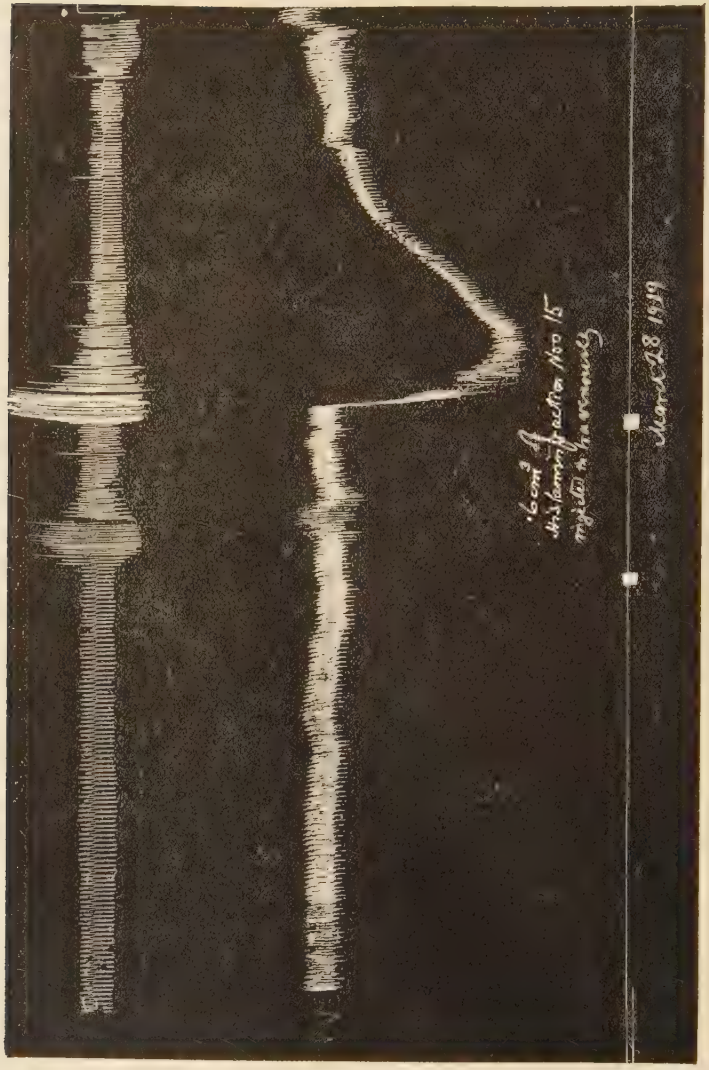


Fig. 1. Respiratory and blood pressure tracings obtained by injecting 0.60 cc. of Histamine fraction No. 15—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

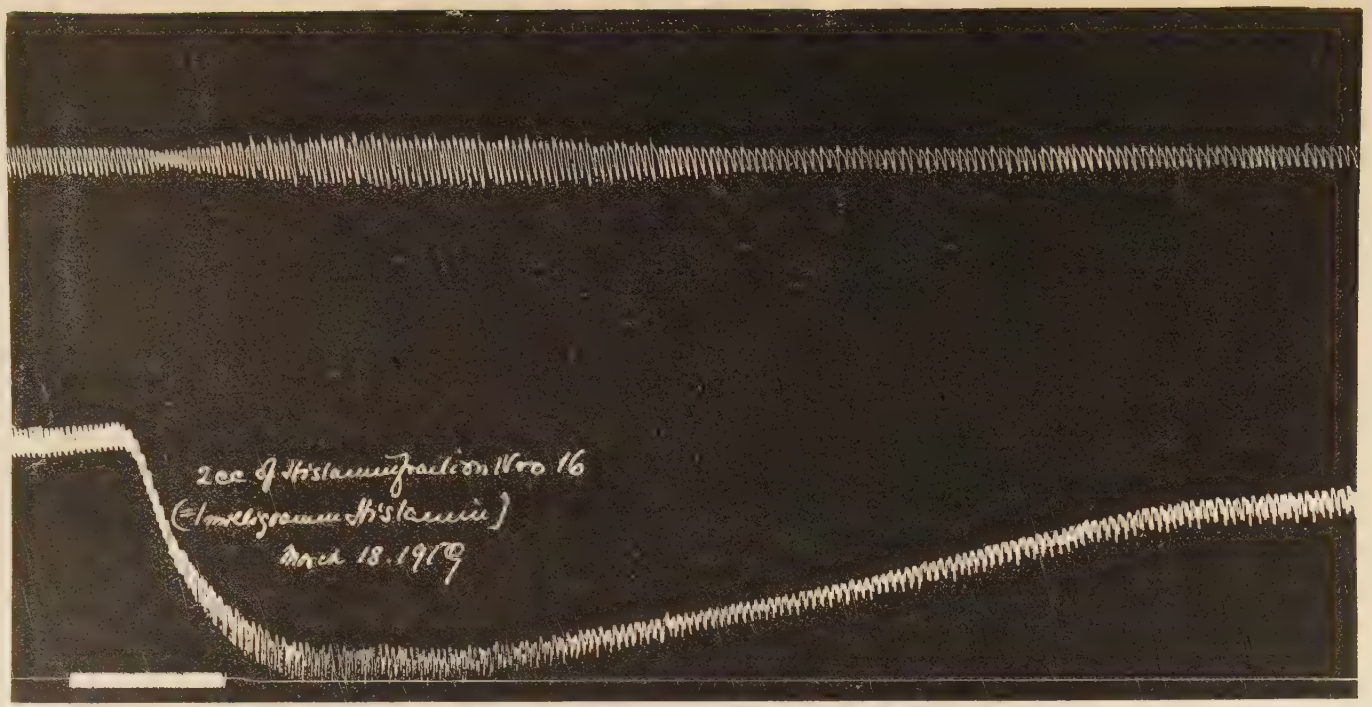


FIG. 2. Respiratory and blood pressure tracings obtained by injecting 2 cc. of Histamine fraction No. 16—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

Chemical Verification of the Presence of Histamine.

The remainder of Solutions 7, 8, 13, 14, 15, and 16, whose combined histamine content was 0.10 gm. by colorimetric determination, was united, made strongly alkaline with sodium hydroxide, and extracted with amyl alcohol in the customary manner. The amyl alcohol was then extracted exhaustively with 6 N HCl. The acid extracts were combined and evaporated on the water bath. An entirely solid, pale brown residue was left which was largely sodium chloride. A melting point determination was carried out on a portion of it. A slight softening occurred at 195°. No further change was noticeable until the temperature had risen to 242° (corrected) when an abrupt softening occurred. The solid did not, of course, melt completely because of the presence of sodium chloride. The recorded melting point of histamine dichloride is 244–246° (corrected.)

The solid gave 0.230 gm. of dipicrate in the form of rhombic leaflets, M.P. 238–240°, from 100 cc. of water. This is the recorded melting point of histamine dipicrate. Some of this solid was mixed with an equal portion of pure synthetic histamine dipicrate. The melting point of the mixture was 238–240°. This proves conclusively that the solid was histamine dipicrate.

A further proof of the quantitative exactness of the colorimetric method can be cited. 0.10 gm. of histamine dichloride should give 0.31 gm. of dipicrate. We have found that the solubility of histamine dipicrate in 100 cc. of water is 0.0716 gm. at 8°, 0.077 at 20°, and 1.4 at 95°. Since this picrate was filtered from a solution whose temperature was about 10°, 0.07 gm. of dipicrate was left in the 100 cc. of solvent. Since 0.23 gm. of dipicrate was actually isolated, 0.30 gm. must have been formed in all which agrees very well with the 0.31 gm. demanded by the theory.

Determination of Ammonia and Total Amino Nitrogen in the Test Solution.

Ammonia was determined by the method given in Section VII of Paper III.

The amino nitrogen determinations referred to here were carried out only on those solutions whose total color value was almost nil. In these cases 10 cc. of the test solution were evapo-

rated on the water bath with 5 cc. of M Na_2CO_3 solution until the liquid was free from ammonia and volatile amines. (These solutions always had a very disagreeable amine-like odor when alkaline which disappeared after the liquid had been heated for some time.) An excess of hydrochloric acid was then added and the liquid reevaporated until all the water and acid had disappeared. The solid was transferred to a precision cylinder with enough water to give a final volume of 10 cc. 5 cc. of this solution were then used for the amino nitrogen determinations. The results are given in Tables IV and V.

TABLE IV.

Ammonia and Total Amino Nitrogen.

Flask No.	0.1 N HCl neutralized by NH_3 from 5 cc. of test solution.	Total nitrogen as NH_3.*	N_2 from 5 cc. of test solution (Van Slyke method).			Total N as amino N.
			Amount.	Temperature.	Pressure.	
	cc.	*per cent*	*cc.*	°C.	*mm.*	*per cent*
1	3.51	66.8	1.75	23	753	13.2
2	1.52	28.9				
3	10.38	82.5	1.97	19	744	6.28 (15.0 per cent of histidine N).
4	6.75	53.8				
5	0.10	1.9				
6	0.10	1.9				
7	3.50	27.9				
8	4.46	35.5				
9	3.95	75.0	1.25	20	748	9.5 (also histidine N).
10	0.50	9.5				
11	8.30	66.2	1.66	20	756	5.35 (12.8 per cent of histidine N).
12	5.80	46.2				
13	0.10	1.9				
14	0.90	17.1				
15	5.68	45.3				
16	6.48	51.7				
17	8.15	65.0				

* N_2 in KNO_3 was ignored.

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid
					pe ce
1. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 90 cc. distilled water.	1.0 cc. = 0.02 mm. Color brown; match poor. **2%**			0
2. Anaerobic.	Same as No. 1.	0.1 cc. = 6.6 mm. 0.2 " = 13.2 " Match perfect. **66.0 %**	0.1 cc. = 6.4 mm. 0.2 " = 12.7 " Match perfect. **64.0 %**	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. **64.5 %**	0
3. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 10 cc. 2 % NH_4Cl. 80 cc. distilled water.	1.0 cc. = 3.5 mm. Color brown; match poor. **3.5 %**			0
4. Anaerobic.	Same as No. 3.	0.1 cc. = 10.1 mm. 0.2 " = 20.3 " Match perfect. **98.0 %**	0.10 cc. = 10.1 mm. 0.20 " = 19.8 " Match perfect. **98.0 %**	1.8 cc. N_2 at 22° and 741 mm. 0.2008 gm. histidine Cl_2. **100.4 %**	0
5. Aerobic.	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 1. 86 cc. distilled water.	0.1 cc. = 9.0 mm. 0.2 " = 18.0 " Match perfect. **90.0 %**	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. **79.0 %**	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. **81.5 %**	0

or value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	17.55	66.8	1.75 cc. N_2 at 23° and 753 mm. 13.2 %.	7.35	8.3
cc. = 3.0 mm. or too yellow; match poor.	≏ 0.0009 gm. histamine in test solution. 0.45 % Histamine absent.	0	7.6	28.9		7.35	7.6
	Histamine absent.	0	51.9	82.5	1.97 cc. N_2 at 19° and 744 mm. 6.28 % total N (15.0 % histidine N).	7.35	8.2
cc. = 4.0 mm. or too yellow; match poor.	≏ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	33.75	53.8		7.35	7.5
cc. = 4.0 mm. or too yellow; match poor.	≏ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	0.50	1.9		7.35	7.15

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into *pe ce*
6. Anaerobic.	Same as No. 5.	0.10 cc. = 10.4 mm. 0.20 " = 20.8 " Match perfect. **104.0 %**	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Too pink. **74.0 %**	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. **58.1 %**	8.
7. Aerobic.	10 cc. 2 % histidine Cl_2. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 76 cc. distilled water.	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. **94.0 %**	0.10 cc. = 4.6 mm. 0.20 " = 9.2 " Match perfect. **46.0 %**	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. **46.0 %**	0
8. Anaerobic.	Same as No. 7.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 6.4 mm. 0.20 " = 12.7 " Match perfect. **64.0 %**	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. **65.5 %**	0
9. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 100 cc. Salt Medium 1. 85 cc. distilled water.	1.0 cc. = 3.7 mm. Color brown; match poor. **3.7 %**			0
10. Anaerobic.	Same as No. 9.	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. **91.0 %**	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. **89.0 %**	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. **89.6 %**	0

olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
0 cc. = 3.8 mm. olor too yellow; match poor.	≏ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	0.50	1.9		7.35	7.45
05 cc. = 11.2 mm. 10 " = 22.2 " olor identical with that obtained with histamine.	≏ 0.075 gm. histamine in test solution. **46.4** % of histamine present.	0	17.5	27.9		7.35	>6.6
10 cc. = 6.6 mm. 20 " = 13.1 " olor identical with that obtained with histamine.	≏ 0.0221 gm. histamine in test solution. **13.7** % of histamine present.	0	22.3	35.5		7.35	>6.6
	Histamine absent.	0	19.75	75.0	1.25 cc. N_2 at 20° and 748 mm. 9.5 % (ignoring KNO_3).	7.35	8.4
0 cc. = 3.8 mm. olor too yellow; match poor.	≏ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	2.5	9.5		7.35	8.0

TABLE

Summary

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference)
					per cent
11. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 100 cc. Salt Medium 1. 75 cc. distilled water.	1.0 cc. = 2.7 mm. Color brown; match poor. **2.7 %**			0
12. Anaerobic.	Same as No. 11.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0 %**	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. **100.0 %**	0
13. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 4 cc. glycerol. 100 cc. Salt Medium 1. 81 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. **40.0 %**	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. **39.5 %**	0
14. Anaerobic.	Same as No. 13.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. **97.0 %**	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. **90.0 %**	1.6 cc. N_2 at 23° and 753 mm. 0.1807 gm. histidine Cl_2. **90.4 %**	0

Continued.

sults. Part I.

olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	41.5	66.2	1.66 cc. N_2 at 20° and 756 mm. 5.35 % total N (12.8 % histidine N).	7.35	8.3
0 cc. = 4.0 mm. olor too yellow; match poor.	≏ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	29.0	46.2		7.35	7.9
05 cc. = 12.4 mm. 10 " = 24.7 " olor identical with that obtained with histamine.	≏ 0.0825 gm. histamine in test solution. **51.0 %** of histamine present.	0	0.50	1.9		7.35	>6.6
20 cc. = 6.4 mm. 40 " = 12.8 " olor identical with that obtained with histamine.	≏ 0.01075 gm. histamine in test solution. **6.7 %** of histamine present.	0	4.5	17.1		7.35	>6.6

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid
					per cent
15. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 71 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0 %**	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0 %**	0
16. Anaerobic.	Same as No. 15.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. **75.0 %**	1.35 cc. N_2 at 20° and 753 mm. 0.1549 gm. histidine Cl_2. **77.4 %**	0
17. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 5 gm. glucose. 100 cc. Salt Medium 1. 70 cc. distilled water.	0.10 cc. = 9.3 mm. 0.20 " = 16.0 " Match good. **93.0 %**	0.10 cc. = 5.3 mm. 0.20 " = 8.2 " Match good. **53%**	1.06 cc. N_2 at 24° and 737 mm. 0.1168 gm. histidine Cl_2. **58.4 %**	0

* Colors matched against the (CR–MO) standard.

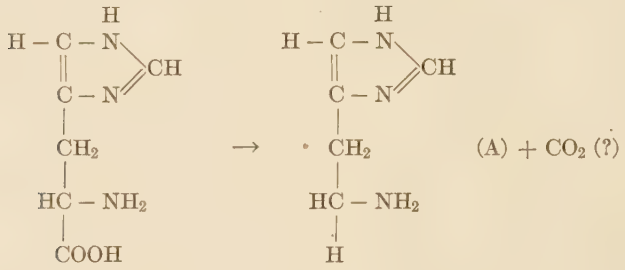
value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
5 cc. = 12.1 mm. " " = 24.2 " r identical th that ob- ined with his- mine.	0.0807 gm. histamine in test solution. **50.0 %** of histamine present.	0	28.4	45.3		7.35	>6.6
0 cc. = 7.5 mm. 0 " = 15.0 " r identical th that ob- ined with his- mine.	0.025 gm. histamine in test solution. **15.5 %** of histamine present.	0	32.4	51.7		7.35	>6.6
0 cc. = 12.0 mm. 20 " = 25.3 " r identical th that ob- ined with his- mine.	0.0425 gm. histamine in test solution. **26.3 %** of histamine present.	0	40.75	65.0		7.35	>6.6

Theoretical Considerations. Discussion of Results.

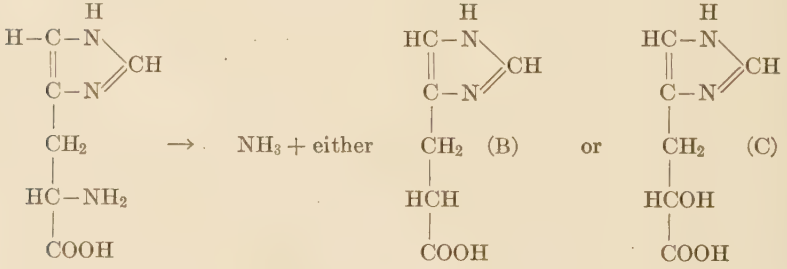
A consideration of the experiments given in Tables IV and V together with those of the other authors referred to in the introduction has led us to the following tentative theoretical deductions.

It should be possible for histidine to undergo four kinds of *primary decomposition.*

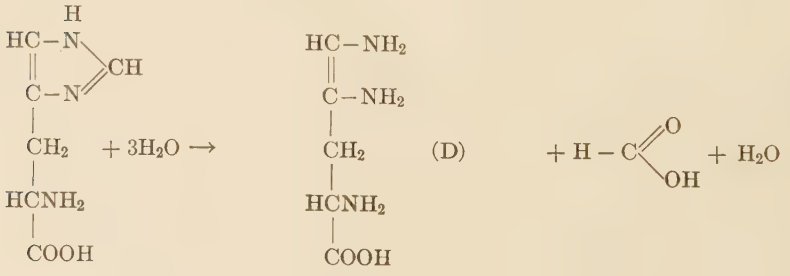
Type 1. Decarboxylation.



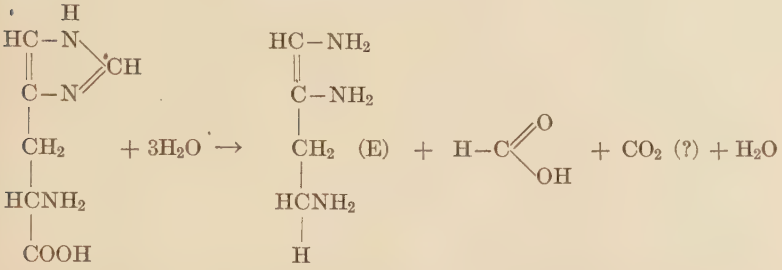
Type 2. Deamination.



Type 3. Nuclear disruption without decarboxylation.

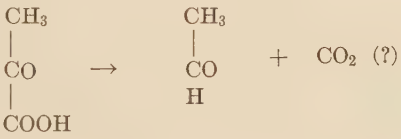


Type 4. Nuclear disruption with decarboxylation.



We have now to consider under what conditions each type of decomposition is to be expected.

Type 1.—We can see only two reasons why a microorganism would decarboxylate histidine: (1) To obtain the particular kind of carbon that is present in the carboxyl group ($\overset{+}{+}C\overset{+}{+}$ but more probably $\overset{+}{-}C\overset{+}{+}$); and (2) to produce the base histamine with which excess of acidity could be neutralized more efficiently than with ammonia. Decarboxylation would lead to a lowering of the hydrogen ion concentration of the solution irrespective of whether a base was formed or not because the organic acids are usually more highly dissociated than carbonic acid. Thus the decarboxylation of pyruvic acid, for example, would lead to a reduction of the hydrogen ion concentration of the solution even though a base was not formed.



We have placed a (?) behind the CO_2 in each case because we are not certain that the carboxyl group appears immediately as CO_2, or, indeed, whether it appears in this form at all. It is possible that formic acid is first formed which later gives CO_2 by oxidation.

This type of decomposition would then be expected when another source of carbon was available and when the hydrogen ion concentration of the medium threatened to become too high for the proper development of the organism.

Type 2.—Histidine might be deaminized for three reasons.

1. The nitrogen might be removed because it was necessary for the proper development of the organism. This would be expected to occur when other available sources of nitrogen, such as ammonium salts or nitrates, were absent. In the presence of an easily available source of carbon but an inadequate source of nitrogen, the decomposition of the histidine molecule might easily cease after the nitrogen had been removed. Under these conditions imidazole lactic or propionic acids might be present unchanged at the end of the experiment.

2. The nitrogen might be removed not because it was needed, but because its removal was a necessary precursor or adjunct to the utilization of the aliphatic carbon chain. Such a decomposition might occur when other available sources of carbon were absent irrespective of the presence or absence of a nitrogen source.

3. Deamination might be resorted to as a means of lowering the hydrogen ion concentration of the medium. The equation given under Type 2 shows that the mere removal of the ammonia from the amino-acid molecule can have but little effect upon the hydrogen ion concentration of the medium because the acid radical is still present and it will neutralize the ammonia at least as completely as it did the amino group. A subsequent oxidation of the aliphatic carbon chain would destroy this acid group, however, and then the ammonia would be of service as a neutralizer of acidity. This process would be a more roundabout method for reducing the pH of a medium than direct decarboxylation. There is little doubt that this process is made use of by the body cells, and some bacteria probably proceed in the same manner.

Types 3 and 4.—A nuclear disruption, either with or without decarboxylation, is the most certain way to expose for future use all the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic. This type of disruption might be expected if either the solution contained no other source of carbon or nitrogen than histidine or the initial pH of the medium was so markedly unfavorable to the organism that it was necessary to liberate a large amount of a base in the shortest possible time. Since a microorganism requires far more carbon than nitrogen for its growth and metabolism, a nuclear rupture would be expected to

occur even in the presence of a large excess of ammonium salts or nitrates, when the medium contained no more available source of carbon than histidine.

It is also to be expected that a substance of Type E, containing no carboxyl group, will pass into the amyl alcohol layer during the extraction process, while one of Type D, containing a carboxyl group, will remain in the alkaline aqueous layer along with the histidine.

We will now pass to a discussion of the individual cases and attempt to explain the results obtained on the basis of these theoretical deductions.

Flask 1.—In this case histidine was the only source of carbon and nitrogen. An examination of Table V shows that (1) the imidazole ring was destroyed to the extent of 98 per cent; (2) about 67 per cent of the total, not amino, nitrogen of the molecule was converted into ammonia; (3) about 13 per cent of the total nitrogen was still in the amino condition; and (4) the pH changed from 7.35 to 8.3. These results can be explained as follows.

The only source of carbon and nitrogen was histidine. A nuclear rupture would expose these elements so that they could serve as foods. Since very little of the nitrogen was required as compared to the carbon, most of it was eliminated as ammonia which lowered the hydrogen ion concentration of the solution.

Flask 2.—The composition of this medium was identical with that of No. 1 except that the oxygen concentration was almost nil. The nucleus was ruptured only to the extent of 34 per cent. The histidine that did not undergo a nuclear cleavage was untouched. About 29 per cent of the total nitrogen appeared as ammonia. The reaction of the medium changed from pH 7.35 to 7.6. The close check between the amino nitrogen and colorimetric values for histidine shows that molecules of Type D were absent. An amino nitrogen determination on the histamine fraction, which was carried out after the solution had been freed from ammonia and volatile amines, showed that 4 per cent of the total nitrogen was present in this form. This indicates that compounds of Type E were present. A nuclear rupture with decarboxylation occurred in this case which proves that *the colon bacillus has the power of decarboxylating acids, which suggests that under proper conditions it may be able to make amines from amino-acids.* All the

nitrogen introduced was accounted for. Thus 66 per cent of the histidine was recovered; 29 per cent of the nitrogen appeared as ammonia; 4 per cent was non-volatile amino nitrogen in the histamine fraction, which makes a total of 99 per cent. In this case, then, volatile amines were absent.

The explanations offered for No. 1 apply also in this case. We have found that the anaerobic differed from the aerobic experiments in quantity, not in quality. The rate of metabolism is much reduced by the oxygen deficit but the quality of the products is not affected.

Flask 3.—The results obtained in this case were almost identical with those obtained for No. 1, with the apparent exception of the ammonia value. Such an amount of ammonium chloride was originally introduced, however, that the ammonia from the entire test solution should have neutralized 36.40 cc. of 0.1 N acid. When this is subtracted from the 51.9 cc. required by experiment, 15.5 cc. of 0.1 N acid are found to have been used to neutralize the ammonia liberated from the histidine which compares very well with the 17.55 cc. required for No. 1.

The analogous results obtained in Nos. 1 and 3 can be explained by the fact that although the organism was supplied plentifully with a nitrogen source, histidine was still the only source of carbon. A nuclear rupture followed by deamination was the surest way to make this carbon available.

Flask 4.—Table V shows that 98 per cent of the histidine was recovered unchanged. The almost complete absence of metabolic activity led us to believe that the organisms had died. This proved not to be the case. This is merely another example of the necessity of oxygen for the metabolism of the colon bacillus.

Flask 5.—In this case histidine was the only source of nitrogen but glycerol was added as a source of carbon. About 80 per cent of the histidine was recovered. Here again there was a complete absence of molecules of Type D. Only 2 per cent of the histidine nitrogen was recovered as ammonia. For the first time we were confronted with a peculiar discrepancy. The total color value determination indicated that about 90 per cent of the imidazole ring was still intact. Only 80 per cent of it was proved to be histidine and the remaining 10 per cent disappeared in the extraction process. We are tempted to conclude that some heterocyclic

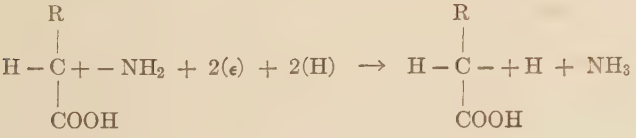
compound of undetermined constitution was present that was completely destroyed by the strong alkali used in the extraction process. This conclusion is rendered still more plausible by the subsequent experiments.

Imidazole lactic, propionic, or acetic acids were absent which, of course, means that they were either not formed under these conditions or subsequently oxidized.

In the presence of an available carbon source the histidine molecule was called upon merely as a source of nitrogen. The nitrogen was obtained partially by deamination and perhaps to a small extent by a partial rupture of the nucleus. The amount of nitrogen liberated exceeded the metabolic requirements of the organism by only 2 per cent.

Flask 6.—The composition of this medium differed from that of No. 5 only in the atmospheric oxygen content, which in this case was almost nil. Of the entire series, this is the one case in which the formation of imidazole propionic acid was indicated. Only 2 per cent of the histidine nitrogen was recovered as ammonia. Here again we were confronted with the same type of color value discrepancy that was noted with No. 5. The total color value determination indicated the presence of 100 per cent of the original imidazole ring. Only 74 per cent of this color value was accounted for as histidine and imidazole propionic acid; about 25 per cent was lost in the extraction process. This again indicates the formation of an alkali-labile heterocyclic compound. A discrepancy of this kind was obtained in only one other case (No. 8) where it amounted to 20 per cent. It seems that the labile compound is formed more easily or destroyed less easily under anaerobic conditions.

The absence of oxygen would hinder oxidation reaction and favor reductions. The formation of imidazole propionic acid from histidine is a reduction regardless of what assumptions are made as to the process.



There is no doubt that, in this case, deamination was resorted to by the colon bacillus as a means of obtaining nitrogen. To judge from the total color value determination, a nuclear disruption did not occur. It appears that this organism is readily capable of rupturing the imidazole ring only in the presence of oxygen.

Flask 7.—This medium contained both an easily available source of carbon and nitrogen. Table V shows that: (1) this is the first member of the series that was distinctly acid after incubation; (2) 46.4 per cent of the histidine was converted into histamine; (3) unchanged histidine was the only other product; therefore (4) a marked nuclear rupture did not occur; and (5) about 50 per cent of the ammonia that was originally introduced was consumed by the bacteria.

The histidine in this medium was unnecessary as a food constituent because the organisms grew perfectly with just the glycerol and ammonium salt to serve as carbon and nitrogen sources. Under these conditions neither a nuclear disruption nor deamination could be expected unless a nuclear rupture was resorted to as a means of neutralizing excess acidity. Decarboxylation occurred to a very marked extent—46.4 per cent—which proves that the colon bacillus has this power. We concluded (see Theoretical Considerations) that the organism would decarboxylate histidine either to get the carboxyl carbon or to manufacture the strong base histamine. This experiment leaves us largely in the dark as to the purpose of the decarboxylation. The fact that histamine was never found, in our experiments, unless the medium had become distinctly acid, suggests strongly that the base was formed merely to lower the hydrogen ion concentration of the solution. In Part III of this paper, proof that this is actually the case is presented.

Flask 8.—This was the anaerobic equivalent of No. 7. Table V shows that (1) the medium was distinctly acid after incubation; (2) 13.7 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) unchanged histidine was the only other recognizable product so that here again there is the indication that an alkali-labile heterocyclic compound was formed to the extent of 20 per cent; (4) there was no evidence of a nuclear disruption; (5) about 40 per cent of the ammonia that was originally introduced was removed from the solution by the bacteria.

The discussion given for No. 7 applies also to this case. The oxygen deficit decreased the rate of metabolism.

Flasks 9 to 16 inclusive.—The remaining experiments were duplicates of those already described except that potassium nitrate was present in all the media. We introduced the nitrate to see whether it would have any effect upon the production of histamine. Potassium nitrate might be expected to function in one or more of the following ways: (1) as a source of potassium or nitrogen; (2) as a source of alkali and ammonia, both of which might be used for the purpose of neutralizing excess of acidity;

$$KNO_3 + 8(H) \rightarrow KOH + NH_3 + 2H_2O$$

(3) as a source of oxygen;

$$KNO_3 + 2(H) + H_2O \rightarrow KOH + NH_3 + 3(O)$$

In this case the ammonia need not, of course, be liberated as such, but might be used for synthetic purposes.

The results obtained in Flasks 13 and 14 prove that KNO_3 is a very efficient source of nitrogen for the colon bacillus. These results were fairly close duplicates of those obtained in Flasks 7 and 8 in which ammonium chloride was present instead of potassium nitrate. A slightly larger percentage of histamine was always formed in the presence of the nitrate than in the presence of the ammonium salt. Thus Flasks 15 and 16 contained more histamine than Flasks 7 and 8 respectively.

In Flask 14, some of the KNO_3 must have been converted into ammonia. In this case 97 per cent of the total nitrogen was present either as histidine or histamine. If all the remaining 3 per cent of nitrogen had been converted into ammonia, the entire test solution would have contained enough of it to neutralize only 0.80 cc. of the 0.10 N acid. Since 4.5 cc. of the acid were actually neutralized by ammonia arising from this solution, we must conclude that enough NH_3 to neutralize 3.7 cc. of the acid must have been formed from the KNO_3 which shows that at least 37 per cent of the nitrate was converted into ammonia.

For the colon bacillus, potassium nitrate seems to serve chiefly as a source of nitrogen. With this in mind the results obtained in Experiments 9, 10, 11, and 12 can be readily understood since they agree perfectly with the theoretical stipulations.

Flask 17.—The nutritive conditions were identical, in this case, to those of No. 15 except that glucose was used as a carbon source in place of glycerol. The results obtained may be summarized as follows: (1) the medium was distinctly acid after incubation; (2) 26.3 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) about 58 per cent of the histidine was unchanged; (4) a nuclear disruption had occurred to the extent of about 15 per cent; and (5) ammonia was generated by the bacteria either from the histidine or from the potassium nitrate. In either case, this ammonia would help to reduce the hydrogen ion concentration of the medium.

Since the nutritive conditions of this experiment were identical with those of No. 15, except for the carbon source, the discovery that 26 per cent of the histidine was converted into histamine would be no surprise if one had not read the literature. The statement is repeatedly made that glucose prevents the decarboxylation of amino-acids by bacteria. Thus Mellanby and Twort,[7] Berthelot and Bertrand,[8] and Jones[11] claim that histamine is not produced in the presence of glucose. The experimental conditions employed by these observers were different from our own and this may account for the difference in results. As will be shown in Parts II and III, such factors as time of incubation and the initial concentration of disodium acid phosphate have a very decided influence upon the formation of histamine from histidine.

There seems to have been a tendency, in the past, to confuse the sparing action of carbohydrates for proteins and the effect of carbohydrates on the decarboxylation of amino-acids. That a body cell or a microorganism will obtain its carbon requirements from carbohydrates by preference to amino-acids or proteins has been proved beyond doubt. Our own experiments show quite conclusively that this principle is correct. Thus in Nos. 1, 3, 9, and 11 where histidine was the only source of carbon, a complete decomposition of the molecule occurred, the histidine being used as a carbon source. When glycerol was added, the histidine remained untouched except as it was called upon to supply nitrogen or to neutralize acidity. The idea that the carbohydrate would also prevent decarboxylation must have arisen because it

[11] Jones, H. M., *J. Infect. Dis.*, 1918, xxii, 125.

was thought that the carboxyl carbon served as a carbon source. If this were actually the case it would be easy to see why the addition of an easily available source of carbon would render decarboxylation unnecessary. The facts are, however, that decarboxylation with amine production occurs only in the presence of an outside source of carbon and that this can be prevented by neutralizing the excess of acidity (see Part III).

These statements apply, of course, only to the colon bacillus. We shall report, at a later date, experiments conducted with other microorganisms and, until these experiments have been completed, a generalization is not admissible.

Part II. The Effect of Duration of Incubation upon Conversion of Histidine into Histamine.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. These experiments were duplicates of No. 15 of Part I except that the time of incubation varied from 2 to 40 days.

The composition of the salt medium did not differ materially from that of Experiment 15. The concentration of the sodium chloride was slightly reduced and the potassium chloride was eliminated. The ammonium chloride and potassium nitrate were incorporated into the salt medium.

Composition of Salt Medium 2.— 4.00 gm. of ammonium chloride.
2.00 " " potassium nitrate.
8.00 " " " dihydrogen phosphate.
16.00 " " sodium chloride.
0.40 " " " sulfate.
8.00 " " " bicarbonate.
0.20 " " calcium chloride.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of Completed Media.—10 cc. of 2 per cent histidine dichloride.
4 " " glycerol.
100 " " Salt Medium 2.
86 " " distilled water.

These amounts were mixed in a 300 cc. Pyrex Florence flask. The salt medium was added from a 100 cc. normal pipette.

The results are given in Table VI.

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into
		days				
1	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 2. 86 cc. distilled water.	2	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. **92.0** %	1.70 cc. N_2 at 22° and 746 mm. 0.191 gm. histidine Cl_2. **95.0** %	
2	Same as No. 1.	5	0.10 cc. = 10.0 mm. 0.20 " = 20.1 " Match perfect. **100.0** %	0.10 cc. = 8.7 mm. 0.20 " = 17.5 " Match perfect. **87.0** %	1.52 cc. N_2 at 22° and 746 mm. 0.1708 gm. histidine Cl_2. **85.5** %	
3	" " " 1.	10	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. **101.0** %	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. **74.0** %	1.38 cc. N_2 at 24° and 737 mm. 0.152 gm. histidine Cl_2. **76.0** %	
4	" " " 1.	14†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0** %	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0** %	
5	" " " 1.	20	0.10 cc. = 11.4 mm. 0.20 " = 23.0 " Color more like that of histamine than histidine. **114.0** %	0.10 cc. = 1.4 mm. 0.50 " = 7.0 " Match perfect. **14.0** %	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5** %	

lor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction.	
						Before incubation	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
0 cc. = 4.0 mm. olor greenish yellow; match poor.	Histamine absent.	0	39.75	63.4		7.35	6.8
40 cc. = 6.7 mm. Match good.	0.0053 gm. histamine in test solution. **3.3** % of histamine present.	0	39.75	63.4		7.35	Slightly >6.6
10 cc. = 11.8 mm. 20 " = 23.5 " Match perfect; identical with that obtained with histamine.	0.039375 gm. histamine in test solution. **24.4** % of histamine present.	0	38.25	61.0		7.35	>6.6
05 cc. = 12.1 mm. 10 " = 24.2 " Match perfect; identical with that obtained with histamine.	0.0807 gm. histamine in test solution. **50.0** % of histamine present.	0	28.4	45.3		7.35	>6.6
025 cc. = 9.4 mm. 05 " = 18.8 " Match perfect; identical with that obtained with histamine.	0.12625 gm. histamine in test solution. **78.0** % of histamine present.	0	31.5	50.2	1.37 cc. N_2 at 24° and 747 mm. 0.1237 gm. histamine Cl_2. **77.0** %	7.35	>6.6

TABLE

Summary

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid
		days				*per cent*
6	Same as No. 1.	30	0.10 cc. = 11.3 mm. 0.20 " = 22.5 " Color more like that of histamine than histidine. **113.0** %	0.50 cc. = 4.0 mm. Match good. **8.0** %	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5** %	0
7	" " " 1.	40	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color more like that of histamine than histidine. **113.0** %	0.50 cc. = 4.0 mm. Match good. **8.0** %	0.33 cc. N_2 at 21° and 751 mm. 0.0377 gm. histidine Cl_2. **18.8** %	0

* Colors matched against the (CR–MO) standard.

† Copied from Table V, Flask No. 15.

Concluded.

sults. Part II.

olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_3 ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
025 cc. = 9.9 mm. 05 " = 19.8 " Match perfect; identical with that obtained with histamine.	0.1325 gm. histamine in test solution. **82.0** % of histamine present.	0	25.5	40.7	1.40 cc. N_2 at 21° and 752 mm. 0.1291 gm. histamine Cl_2. **80.0** %	7.35	>6.6
025 cc. = 10.1 mm. 05 " = 20.2 " Match perfect; identical with that obtained with histamine.	0.135 gm. histamine in test solution. **83.5** % of histamine present.	0	20.0	32.0	1.53 cc. N_2 at 24° and 752 mm. 0.139 gm. histamine Cl_2. **86.0** %	7.35	>6.6

Although the table is largely self explanatory, the following facts call for special emphasis.

The organisms multiplied abundantly during the first 2 days. Some acid was produced but not enough to counteract the neutralizing effect of the $KNaHPO_4$. Ammonia was formed either from the nitrate or from the histidine. *Histamine was absent.*

During the next 3 days enough acid had been formed to counteract entirely the neutralizing ability of the phosphate and the generated ammonia. *During this period 3.3 per cent of the histidine was converted into histamine.*

During the next 5 days decarboxylation proceeded rapidly so that a total of 24.4 per cent of the histidine was converted into histamine. The concentration of ammonia began to drop as it was used to supply the nitrogen requirements of the organism.

During the next 10 days there was an enormous increase n the concentration of the histamine so that 78 per cent of the histidine had been decarboxylated during the entire 20 day period.

The next 20 days produced a slight but steady increase in the histamine concentration and a marked decrease in the ammonia concentration. During this period the process of decarboxylation, as a means of neutralizing acidity, seems to have given place to the far more efficient process of nuclear disruption; for although the color value determination indicated the presence of only 8 per cent of histidine, the amino nitrogen determination showed that the equivalent of 18 per cent of histidine was present. This shows that about 3 per cent of the histidine had undergone a nuclear rupture with the formation of a compound of Type D (see p. 564). The absence of appreciable quantities of compounds of Type E was proved by the close check between the colorimetric and the amino nitrogen determinations on the histamine fraction. A rupture of the imidazole ring in the histamine molecule did not occur during these 40 days of incubation.

Finally, we wish again to call attention to the fact that histamine appeared only in the media that had become distinctly acid during the incubation period.

Part III. Histamine May Be Formed by the Colon Bacillus to Lower the Hydrogen Ion Concentration of the Medium.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. The composition of the salt medium was changed so that the glycerol became a part of it, which made it unnecessary to add this substance separately. We repeat the composition of this complete medium so that it may be referred to conveniently in our later reports.

Composition of Nutritive Medium 3.— 4.00 gm. of ammonium chloride.
2.00 " " potassium nitrate.
8.00 " " " dihydrogen phosphate.
16.00 " " sodium chloride.
0.40 " " anhydrous sodium sulfate.
8.00 " " sodium bicarbonate.
0.20 " " anhydrous calcium chloride.
80.00 cc. " glycerol.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of the Completed Media.—

No. 1. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
2.5 gm. of $Na_2HPO_4.12\ H_2O$.

No. 2. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
3.8 gm. of $Na_2HPO_4.12\ H_2O$.

No. 3. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
0.50 gm. of sodium formate.

No. 4. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
1 " " 2.5 per cent filtered litmus solution.

DISCUSSION.

The four flasks were incubated at 37° for 14 days. Flask 4 was equipped with a two-hole rubber stopper into which were inserted a dropping funnel and a glass tube that served as an air vent. During the 2 week period, 14 cc. of normal sodium hydroxide and 2 cc. of sterile 2.5 per cent litmus solution were added to the contents of the flask through the funnel. The reaction of the medium was slightly toward the acid side of neutrality all the time and it became distinctly acid a few times. The addition of the litmus solution was necessary because the bacterial action discharged the color of the mixture entirely.

An abundant growth was obtained in all the flasks. The bacteria were alive at the end of the experiment. None of the media was contaminated.

The litmus that remained in Flask 4 at the close of the experiment did not interfere with the colorimetric determinations because practically all of it appeared in the form of a coagulum when the filtered liquid was evaporated with 1 cc. of 95 per cent sulfuric acid.

The results are given in Table VII.

The following facts call for special emphasis and interpretation.

The media employed in these experiments were identical with those of Part II of this paper, except that various sources of alkali were added. In Experiment 4 of Part II, the time of incubation was 14 days which makes it analogous to the experiments now under consideration. In this time interval 50 per cent of the histidine had been converted into histamine.

In Experiment 1 of Part III, where 2.5 gm. of hydrated basic sodium phosphate were added to control the pH of the medium, only 10 per cent of the histidine had been converted into histamine. When, in Experiment 2, the concentration of the phosphate was increased so that a total of 3.8 gm. had been added, only 4 per cent of the histidine was converted into histamine. Some free acid had accumulated in both cases which shows that not enough basic phosphate had been added to neutralize entirely the accumulating acid.

From these experiments one would be tempted to conclude that histamine was produced only after the neutralizing effect of the basic phosphate had been overcome by the accumulating acid.

The initial addition of more phosphate would keep the solution neutral for a longer time; hence would delay the production of histamine. This would seem to indicate strongly that the base histamine was produced from histidine to reduce the pH of the solution. When basic phosphate was added, the production of the amine was rendered unnecessary for a time because it, the phosphate, prevented a rise in the pH of the medium.

It might be claimed, however, that in some way the phosphate inhibited the decarboxylase activity of the microorganism; therefore, Experiment 4 was carried out. In this case sodium hydroxide was added from time to time to keep the solution as nearly neutral as possible. Although a luxuriant growth was obtained, only 1 per cent of the histidine was decarboxylated under these conditions. There can be no doubt, then, that in this case the neutralization of the medium prevented, or, as we might say, rendered unnecessary, the production of histamine.

Experiment 3 was originally carried out to see if the addition of carboxyl carbon in an easily available form would prevent the decarboxylation of histidine. The addition of formic acid in any but very small concentrations was not feasible because it would raise the pH of the medium to such an extent that the organisms would be unable to multiply properly. Sodium formate was, therefore, resorted to. We soon saw, however, that this experiment would not prove the point for the following reasons.

Omelianski[12] has shown that sodium formate is rapidly decomposed by *Bacterium formicum* according to the equation

$$2(HCOONa + H_2O) \rightarrow Na_2CO_3 + H_2O + CO_2 + 2H_2.$$

Pakes and Jollyman,[13] and Karczag and Schiff[14] have shown that formic acid is similarly decomposed by the colon bacillus. Since this decomposition is one that proceeds readily and rapidly, sodium formate is really an excellent source of alkali since it yields sodium carbonate when it is acted upon by bacteria. Instead of decarboxylating histidine to obtain the base histamine, sodium formate was decarboxylated which gave rise to the very

[12] Omelianski, W., *Centr. Bacteriol., 2te Abt.*, 1904, xi, 177, 256, 317.

[13] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[14] Karczag, L., and Schiff, E., *Biochem. Z.*, 1915, lxx, 329.

Flask No.	Composition of completed medium; incubated for 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
1	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 2.5 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Match perfect. **93.0** %	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match perfect. **76.0** %	1.4 cc. N_2 at 22° and 745 mm. 0.1575 gm. histidine Cl_2. **78.8** %
2	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 3.8 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. **86.0** %	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. **83.0** %	1.5 cc. N_2 at 26° and 754 mm. 0.167 gm. histidine Cl_2. **83.5** %
3	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 0.5 gm. sodium formate.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.2 mm. 0.20 " = 18.2 " Match perfect. **92.0** %	1.73 cc. N_2 at 27° and 747 mm. 0.1895 gm. histidine Cl_2. **95.0** %
4	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 1 cc. 2.5 % filtered litmus solution.†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0** %	1.77 cc. N_2 at 26° and 754 mm. 0.1974 gm. histidine Cl_2. **98.7** %

* Colors matched against the (CR–MO) standard.

† See special record, page 580.

‡ This is probably high by 0.6 per cent due to admixture with histidine.

olor value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_2 ignored).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
10 cc. = 5.0 mm. 50 " = 25.1 " atch perfect; identical with that obtained with histamine.	0.0165 gm. histamine in test solution. **10.2** % of histamine present.	0	23.0	36.7	0.22 cc. N_2 at 22° and 750 mm. 0.02011 gm. histamine Cl_2. **12.5** %	7.5	>6.6
50 cc. = 10.2 mm. 0 " = 20.2 " atch perfect; identical with that obtained with histamine.	0.0068 gm. histamine in test solution. **4.2** % of histamine present.	0	23.5	37.5	0.06 cc. N_2 at 30° and 752 mm. 0.00527 gm. histamine Cl_2. **3.25** %	7.7	Slighlty >6.6
0 cc. = 9.7 mm. atch good for histamine.	0.00325 gm. histamine in test solution. **2.0** % of histamine present.	0	34.7	55.3		7.4	6.6
0 cc. = 5.7 mm. atch good; more like histidine than histamine.	0.00175 gm. histamine in test solution. **1.08** %‡ of histamine present.	0	18.5	29.5	0.02 cc. N_2 at 30° and 752 mm. 0.001758 gm. histamine Cl_2. **1.09** %	Medium kept approximately neutral to litmus by addition of N NaOH.†	

much more efficient acid neutralizer sodium carbonate. In this case then, only 2 per cent of the histidine was converted into histamine.

SUMMARY.

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.